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THE RESULTS OF INOCULATING PINUS STROBUS WITH THE SPORIDIA OF CRONARTIUM RIBICOLA[1]

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INTRODUCTION

During the rather extensive field investigations of white pine blister rust which the Office of Forest Pathology conducted at North Conway, N. H., from 1918 to 1922, large numbers of *Pinus strobus* L. were inoculated with sporidia of *Cronartium ribicola* Fischer. Very little infection resulted from the early inoculations, but a great deal was noted after certain changes in inoculation technique in 1921 and 1922. Preliminary reports on the 1921 inoculations of potted pines (*17*) and on the 1922 inoculations of pines in situ (*7*) have been published. In this latter article various attempts of other investigators (*1, 2, 6, 8, 16, 17*) to infect pines with sporidia artificially are discussed. In the present paper the results of the 1921 and 1922 inoculations of potted pines and the 1925 observations on the 1922 inoculations of pines in situ are reported.

METHODS AND CONDITIONS OF INOCULATION

The methods of inoculation used during the summers of 1921 and 1922 and the conditions under which the inoculations were made, simulated various natural conditions as closely as possible. In both years potted seedlings of *Pinus strobus* ranging in age from 1 to 10 years (most of them under 5 years) and in 1922 pines in situ ranging in age from 5 to 30 years, all of which were growing under natural conditions in the woods, were inoculated with sporidia of *Cronartium ribicola*. All inoculations were made in areas from which the Ribes had been eradicated in 1916. The trees and seedlings inoculated had grown in these areas. This reduced to a minimum the danger of natural infection of the inoculated pines as well as the danger of disseminating the disease from them to near-by Ribes and thence to uninoculated pines. The potted seedling pines had been carefully dug up and transplanted in paraffin paper cups some time before inoculation, so that they had become well established in the pots and had reached a state of physiological equilibrium. The pots had been kept from the time of transplanting to the time of inoculation near a brook in a shady swamp, except in the indicated experiments.

Inoculating material was obtained in 1921 from *Ribes nigrum* L., which was growing in two different localities, and in 1922 from *R. nigrum*, in the same localities, and from *R. odoratum* Wendl. and *R.*

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[2] The writers are indebted to Perley Spaulding for field examinations of the inoculated seedlings in November, 1921, to Glenn G. Hahn for assistance in making some of the 1922 inoculations, and to R. H. Colley and Miss M. W. Taylor for sectioning and diagnosing the disease of some of the infected seedlings.

cynosbati (L.) Mill. The inoculum was selected with great care, new generations of telial columns being collected whenever possible.

In both 1921 and 1922 all potted seedlings were inoculated in iceless refrigerators similar to that described by Hunt (*5*). Some of the pines in situ were inoculated in iceless refrigerators and some on rainy days without them. The temperatures and relative humidities at which the experiments were conducted were recorded by means of frequently checked Fries hygrothermographs.

In 1921 four methods of applying the inoculum to the pine needles were used: (1) An iceless refrigerator was set over a *Ribes nigrum* bush which was producing telia in great abundance. The bush was sprinkled, and potted pine seedlings which had previously been sprayed were placed under it. Throughout the inoculation period droplets of water were present on both the Ribes bush and on the seedling pines. With the exception of this period, the pines were kept more than 900 feet from any Ribes. (2) Telium-bearing Ribes leaves were cut into pieces (Table 1) which were supported by small sticks above the needles of the pines before the teliospores had begun to germinate. (3) Telium-bearing Ribes leaves were cut into pieces (Table 1) and the teliospores were allowed to germinate in Petri-dish moist chambers which were kept on the ground in a cool, moist, shaded spot in the woods for six hours. At the end of that period the pieces of Ribes leaves were supported above the pine needles as in method 2. (4) Telial columns were floated on the surface of water in small glasses which were covered with half Petri dishes and kept under conditions similar to the Petri-dish moist chambers of method 3. At the end of six hours small quantities of germinated telial columns together with the sporidia were removed from the glasses and placed in needle fasicles but not smeared around on the needles. The potted pines inoculated by methods 2, 3, and 4 were sprayed just before and just after the inoculum was applied. They were placed immediately in an iceless refrigerator. For all experiments freshly collected Ribes leaves were used. The inoculum remained on the seedlings for the periods given in Table 1. In some experiments immediately after the removal of the inoculum the seedlings were placed outside in the swamp and in others they were covered with paraffin paper cups to prevent infection from inoculum on other seedlings and were left in the iceless refrigerator in order to continue conditions favorable for infection.

In two experiments the effect of drought and of high humidity upon susceptibility to infection were compared. In these experiments part of the seedlings were exposed to conditions of extreme drought and the others to very high humidity for about a week before inoculation. The seedlings used in the first experiment were placed in half shade and were allowed to wilt frequently, being watered only enough to prevent them from dying. They had almost reached the wilting point when inoculated. The seedlings in the second experiment were set in the shade and the soil in which they were growing was kept almost saturated. They were placed in the iceless refrigerator for several hours before inoculation. After inoculation the seedlings which had received both treatments were placed in the iceless refrigerator. Details of the 1921 experiments are given in Table 1.

TABLE 1.—*Inoculations of potted pines made in 1921 (results to October, 1925)*

Conditions of inoculation					Results of inoculation						
					First-year seedlings		Older seedlings		All seedlings		
Date of inoculation (1921)	Pregermination of teliospores [a]	Amount of inoculum	Number of hours inoculum remained on seedlings [b]	Number of hours seedlings remained in iceless refrigerator [c]	Number inoculated	Per cent infected	Number inoculated	Per cent infected	Number inoculated	Per cent infected [d]	Per cent living and uninfected [d]
Aug. 12	None	Ribes bush	9¼	9¼	3	0	2	0	5	0	60
Do	do	do	9½	21	0	------	5	0	5	0	60
Do	do	do	14½	21	0	------	6	17	6	17	50
Do	do	do	36	36	8	63	14	14	22	32	59
Aug. 16	do	Leaf fragments 3 to 10 cm. square.	6	42	0	------	4	25	4	25	25
Do	do	do	17	41	3	100	3	33	6	67	33
Do	do	do	24	24	3	67	4	100	7	86	14
Do	do	do	36	36	12	100	26	96	38	97	3
Aug. 25	By floating	Small quantity telia and sporidia.	24	24	2	50	12	25	14	29	50
Do	None	Leaf fragments ½ to 1 inch square.	21	21	1	100	18	44	19	47	37
Aug. 26	do	Leaf fragments 1 inch square	6	6	0	------	5	40	5	40	60
Do	do	do	9	9	0	------	4	25	4	25	75
Do	do	do	9	51	1	100	4	50	5	60	40
Do	do	do	42	42	18	78	11	82	[e]29	79	10
Aug. 24	In Petridish	do	23½	23½	3	67	9	78	12	75	8
Aug. 29 [f]	do	do	24	24	4	50	7	57	11	55	9
Do [g]	do	do	24	24	2	100	8	25	10	40	40
Do [f]	By floating	Small quantity telia and sporidia.	24	24	0	------	6	67	6	67	17
Do [g]	do	do	24	24	0	------	9	67	9	67	33

[a] In all experiments in which teliospores were pregerminated (germinated before being placed in iceless refrigerator) the germination period was six hours.

[b] Some seedlings were placed outside immediately after the removal of the inoculum while others were covered with paraffin paper cups and kept in the iceless refrigerator.

[c] When the seedlings were removed from the iceless refrigerator they were placed outside in the swamp.

[d] It will be noted that in some cases the percentages in the last two columns do not total 100. This is because some of the seedlings died before it was possible to determine either the presence or the absence of infection.

[e] Includes five plants on which inoculum remained only six hours but which were accidently mixed with others.

[f] Exposed to extreme drought for about one week before inoculation.

[g] Exposed to high humidity for about one week before inoculation.

In 1922 the methods of inoculation were very similar to Nos. 2 and 3 which were used in 1921. After collection, the telium-bearing Ribes leaves were placed in a refrigerator for at least 12 hours, and the leaves were then cut into quarters or halves according to size. In part of the experiments the telial columns were ungerminated and in the others they had been allowed to germinate in Petri-dish moist chambers which were kept in the laboratory for six hours before being carried to the above-mentioned swamp. The pieces of telium-bearing Ribes leaves were lodged in the axils of the pine needles where they were held loosely in place by means of hairpins made of fine wire (*7, fig. 2*) instead of by means of small sticks as in 1921. The seedlings were sprayed before and after inoculation and were placed immediately in iceless refrigerators, as in 1921. The inoculum was left in place for varying periods. (Table 3.) After the removal of the inoculum some seedlings were immediately placed outside in the swamp, while others were placed for varying periods in a second iceless refrigerator instead of being covered by paraffin paper cups in the same iceless refrigerator as in 1921. In both 1921 and 1922 all

inoculating materials were buried just as soon as they were removed from the seedlings.

In both years after the inoculated seedlings were removed from the iceless refrigerators they were left in the swamp for a few days where they were shaded all day. They were then placed for a while in a place where they received half shade. Finally they were planted in a garden where the pots were buried in the soil. The bottoms of the pots were perforated or removed before planting. The seedlings were watered frequently until they became established. Uninoculated seedlings were planted in the garden beside the inoculated ones for the purpose of securing additional evidence that the infections on the inoculated seedlings had resulted from artificial rather than natural inoculations.

RESULTS OF THE 1921 INOCULATIONS OF POTTED PINES

The results of the 1921 inoculations are reported in one section and those of the 1922 inoculations in the section immediately following, because the 1922 results did not confirm those for 1921 in certain details.

Data were taken on the 1921 inoculations in November, 1921 (*17*), in July, 1922, in June, 1923, and in June and October, 1925. The unavoidably long intervals between observations make it probable that some infected seedlings died without recognition of the infection. No seedling was classified as infected unless definite evidence of infection was obtained. The presence of infection was determined by the external symptoms and in certain doubtful cases by sectioning and staining by the method described by Colley (*3*).

The first external symptoms of white pine blister rust infection are the characteristic yellow spots in the needles described by Clinton (*2*) and illustrated for the first time in color in Plate 1 of this article. These had appeared in the needles of some of the seedlings three months (*17*) after inoculation. Subsequent symptoms of infection were the stunting of growth, the swelling of the stem, the discoloration of the stem, the curving of the stem, abnormally bushy growth, and the production of adventitious buds. Usually a seedling showed more than one of these symptoms.

For each experiment performed in 1921 the percentage of inoculated seedlings which showed evidence of infection between the time of inoculation and October, 1925, is given in Table 1. No infection was noted on any of the uninoculated seedlings, although it was present in every series of inoculated seedlings with the exception of two. It occurred even when the inoculum was removed and the seedlings were set out of doors in the swamp six hours after ungerminated telial columns were suspended over them in the iceless refrigerator. The purpose of this experiment was to ascertain whether infection may occur in nature at the time of heavy dews. The results seem to indicate that such infections would be possible. The minimum time needed for white pine blister rust infection is made by this result to appear materially shorter than the 17½ hours previously suggested (*12*).

EXPLANATORY LEGEND FOR PLATE 1

Seedling of *Pinus strobus*, showing the yellow needle spots characteristic of white pine blister rust infection

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It is well known that during some years blister rust infections on pines are abundant, while during others in which Ribes infections are seemingly just as abundant there is little or no infection of pines. The fact that in one experiment sporidia were observed three hours after teliospores were placed in conditions suitable for germination (*14*), that sporidia began to germinate one hour after being placed under suitable conditions (*17*), that the infection period is sometimes as short as recorded in Table 1, and that dried sporidia may sometimes remain viable for 26 hours (*14*), indicate that the physical factor requirements of the fungus are less exacting than has been supposed. It is true that no tests have been made to determine whether the sporidia which were observed after three hours were mature enough to germinate, and none have been made to determine whether the sporidia found viable after long periods of drying are still capable of infecting pines. The information available

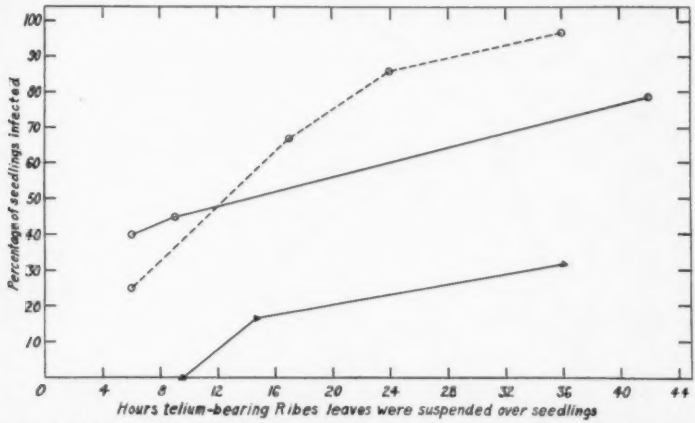

FIG. 1.—Relationship in the 1921 experiments between the amount of infection and the length of time the inoculum remained on the seedlings. Each curve represents a different experiment. The teliospores were ungerminated when suspended over the seedlings

seems, nevertheless, to warrant the suggestion that the internal condition of the pines may be quite as important as external physical factors in explaining the infrequence of pine infections.

In the 1921 experiments the amount of infection tended to increase directly with the length of time the inoculum remained on the seedlings. (Fig. 1 and Table 1.) This relationship was less evident for the 1922 experiments than for those of 1921; and in the 1921 experiments less for first-year seedlings than for older ones. It is probable that the longer the teliospores were suspended over the seedlings the more sporidia were produced and hence the greater the chances of infection. Of course it is possible that the observed relationship was purely accidental and resulted from the difference in susceptibility of individual trees or individual needles. The fact that the relationship for the first-year seedlings was less evident than for the older ones may possibly be explained on the ground of the greater susceptibility of the first-year seedlings, that is, the first-year seedlings were so susceptible that they became infected regardless of the

length of time that the inoculum remained upon them. At any rate in comparable 1921 experiments 77 per cent of the first-year seedlings became infected, whereas only 58 per cent of the older ones (most of which were over 3 years old) became infected. Clinton (*2*) found that a larger percentage of his 1-year-old than of his older seedlings became infected.

The two experiments conducted for the purpose of determining the comparative effect of exposure of seedlings of *Pinus strobus* to drought and to high humidity for some time before inoculation upon their subsequent susceptibility to infection with sporidia of *Cronartium ribicola* were inconclusive. Seedlings exposed to drought as well as those which had been kept at a high relative humidity became

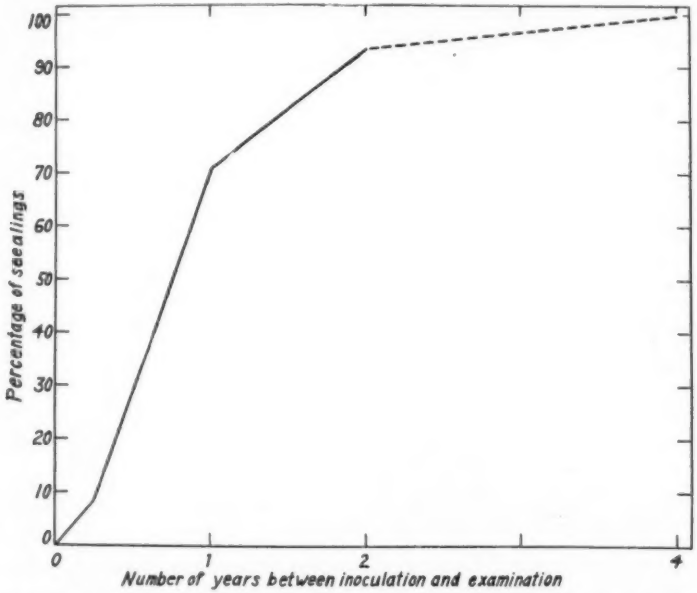

FIG. 2.—Rate at which external symptoms of infection appeared on the infected seedlings in the 1921 inoculations. No data were taken three years after inoculation

infected, and there was not enough difference between the amounts of infection to show which condition made the seedlings more susceptible. (Table 1.) However, in October, 1925, it was found that in both experiments fewer seedlings were living and uninfected among those which had been exposed to drought just before inoculation than among those kept at high relative humidity. Of course it is possible that the exposure to drought and not rust infection may have been responsible for some of these deaths.

Some infections resulted from all four methods of inoculation. (Table 1.) More infections were obtained with sporidia produced by teliospores which had been germinated by "floating" upon the surface of water than in previous attempts by this method, and

more than by inoculation at the same time and under the same conditions with sporidia produced in moist chambers. It is likely that the reason for the greater number of infections is that more sporidia are produced by "floating" than by germination in moist chambers (*13*). In earlier tests without iceless refrigerators few infections resulted from inoculations with sporidia produced by "floating."

The rate at which external evidences of infection from the 1921 inoculations became visible in the infected seedlings is shown in Figure 2. Most of the infections which had occurred had become visible within two years after inoculation. One year after inoculation about 14 per cent of the infected seedlings (fig. 3) had died and

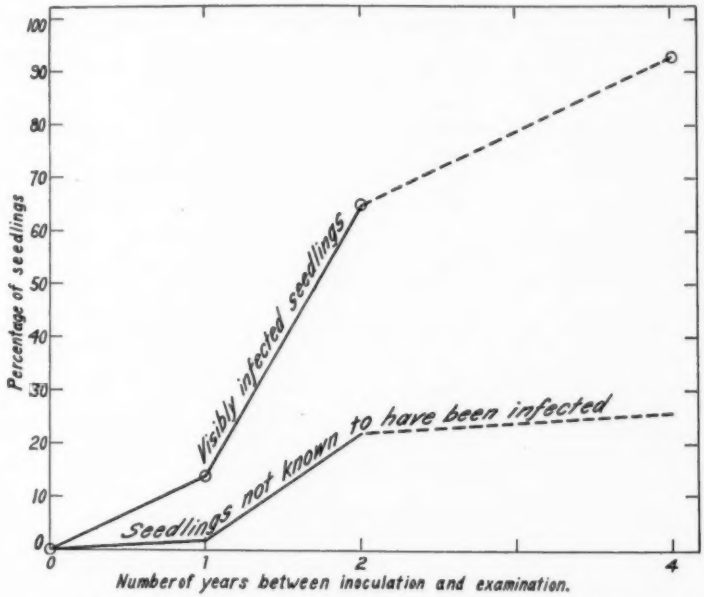

FIG. 3.—Death rate of the visibly infected seedlings inoculated in 1921 and of those not known to have been infected

about one-third of them showed external symptoms of blister rust in their woody parts.

Figure 3 gives a comparison between the rate of death of the seedlings known to have been infected and of those not known to have been infected. Without doubt some of the latter were really infected, since some of them had died and disappeared before they were examined for evidences of infection. If all infections had been detected before death the differences between the two lines in Figure 3 would probably have been still greater. No leaf spots were detected on some infected seedlings while leaf spots, but no further evidence of infection were found on other seedlings. These latter were not classified as infected.

RESULTS OF THE 1922 INOCULATIONS OF POTTED PINES

Data were taken on the 1922 inoculations of potted pines in June, 1923, and in June and October, 1925. The results are given in Tables 2 and 3. The unavoidably long intervals between observations made it inevitable that a great many seedlings should die without either positive evidence of infection or of lack of it having been obtained. There was no consistent relationship between these deaths and the length of time the inoculum remained on the seedlings or between the deaths and the age of the seedlings. There was, however, a tendency for larger percentages of the younger than of the older seedlings to die.

TABLE 2.—*Amount of infection on potted seedlings of different ages (1922 inoculations)*

TELIOSPORES UNGERMINATED WHEN SUSPENDED OVER SEEDLINGS

	Age of seedlings									
	11th year	8th year	7th year	6th year	5th year	4th year	3d year	2d year	1st year	Total
Number of seedlings inoculated	.....	1	1	8	10	13	10	15	3	61
Percentage of seedlings dead, 1923	.....	100	0	13	20	15	30	40	33	26
Percentage of remainder with doubtful results [a]	.....	.....	0	14	25	45	57	55	50	40
Percentage of remainder infected	.....	.....	0	43	50	45	14	11	0	31

TELIOSPORES GERMINATED WHEN SUSPENDED OVER SEEDLINGS

Number of seedlings inoculated	2	3	6	19	38	42	28	82	9	229
Percentage of seedlings dead, 1923	0	0	0	0	5	5	29	34	43	24
Percentage of remainder with doubtful results [a]	0	0	50	21	22	40	30	52	100	38
Percentage of remainder infected	100	67	16	32	42	38	20	24	0	31

[a] Most of these seedlings died between June, 1923, and June, 1925.

TABLE 3.—*Amount of infection on potted seedlings on which inoculum had been suspended for different periods (1922 inoculations)*

TELIOSPORES UNGERMINATED WHEN SUSPENDED OVER SEEDLINGS

	Inoculum in place									
	5 to 6 hours	9 to 10 hours	11 to 12 hours	18 to 19 hours	24 hours	27 hours	30 hours	48 hours	68 hours	Total
Number of seedlings inoculated	.....	.....	18	14	14	.....	7	8	.....	61
Percentage of seedlings dead, 1923	.....	.....	22	21	43	.....	43	0	.....	26
Percentage of remainder with results doubtful [a]	.....	.....	50	54	13	.....	50	24	.....	40
Percentage of remainder infected	.....	.....	21	27	50	.....	25	38	.....	31

TELIOSPORES GERMINATED WHEN SUSPENDED OVER SEEDLINGS

Number of seedlings inoculated	54	24	42	35	44	10	.....	11	9	229
Percentage of seedlings dead, 1923	17	13	26	20	19	20	.....	9	22	24
Percentage of remainder with results doubtful [a]	47	47	29	43	17	37	.....	60	43	38
Percentage of remainder infected	29	29	32	25	49	13	.....	20	29	31

[a] Most of these seedlings died between June, 1923, and June, 1925.

In 1922 some infection occurred in every experiment. Contrary to the 1921 results, the amount of infection seemed to bear no consistent relationship to the length of time in which the inoculum remained in place. Sporidia from teliospores which were ungerminated when suspended over the seedlings produced on the whole as much infection as those from teliospores which were pregerminated. The infection percentages of seedlings which were placed out of doors after the removal of the inoculum were approximately the same as those of seedlings upon which the inoculum had remained for an equal length of time but which were placed in a second iceless refrigerator after its removal. However, the one 1921 experiment in which like comparisons were possible indicated a very decided advantage for continuation in the iceless refrigerator after the removal of the inoculum.

Seedlings of all years except first-year ones became definitely infected, contradicting the 1921 results that seedlings of the current year were more readily infected than older ones. This fact, together with the fact that two-thirds of the seedlings infected in 1921 had died within two years of infection and 90 per cent within four years, helps to account for the absence of 1 to 15 year old seedlings (*4*) in stands infected with blister rust at Waterford, Vt., and at other places.

SUPPLEMENTARY OBSERVATIONS ON THE 1922 INOCULATIONS OF TREES IN SITU

The conditions and methods of inoculating 5 to 30-year old pines in situ in the summer of 1922, and the results of these inoculations which had appeared by September, 1924, have been published (*7*). In 1925 only a portion of the 1922 inoculations could be found because during logging operations some of the inoculated trees had been broken down and destroyed and labels had been removed from others. For this reason the results given below are less representative than they otherwise would have been. From Table 4, it can be seen that there is no real evidence to support the suggestion (*7*) previously made that infection occurred less readily within moist chambers than outside of them on rainy days. For the different experiments the percentage infected ranged from 0 to 90 of the inoculated branches. On these branches the percentage of inoculated twigs infected ranged from 0 to 100. Even on the infected branches only 24 per cent of the twigs were infected.

TABLE 4.—*Results of the 1922 inoculations of 5 to 30 year old pines growing naturally in the woods (observations made in October, 1925)*

Place of inoculation	Number of branches inoculated	Number of branches infected	Per cent of branches infected	Number of twigs inoculated	Number of twigs infected	Per cent of twigs infected
In moist chambers	* 126	39	31	823	103	13
Without moist chambers	* 40	14	35	374	37	10
Total	* 166	53	32	1,197	140	12

* As a rule several twigs were inoculated on each branch. The figures in column 2 differ from those reported for the September, 1924, (*7*) observations because a great many inoculated branches were destroyed during logging operations.

Various symptoms of blister rust were present on the infected twigs in 1925. These consisted of discolorations, swelling, constriction, and deformation (curving) of the twigs, and pycnial and aecial scars. Frequently more than one symptom was present on the same twig. Many of the cankers had girdled the infected twigs and killed the distal parts of them. Many of the killed parts had been invaded by secondary fungi. One small tree about a foot high which was infected in every twig had been killed before June, 1925. In June and September, 1923, practically the only symptoms of blister rust infection observed were the characteristic needle spots (*2*).

No symptoms of infection have been observed on the nine trees of *Pinus resinosa* Ait., which were inoculated with sporidia of *Cronartium ribicola* by the same method as the *P. strobus* trees (*7*) both with and without moist chambers. Clinton (*2*) also got negative results with this species.

RELATION OF TEMPERATURE AND RELATIVE HUMIDITY TO INFECTION OF PINES

So many factors influence the infection of *Pinus strobus* by the sporidia of *Cronartium ribicola* that it is often impossible to determine whether the observed temperatures and relative humidities or some undetected causes are responsible for the failure of inoculations. Temperature and relative humidity influence the production and the germination of the sporidia as well as the actual entrance into the pine needles.

Teliospores will germinate and produce sporidia when floating upon the surface of water and in moist chambers at temperatures of 55° to 80° F. Spaulding (*10*) also found them germinating upon melting snow.

Sporidia germinated in iceless refrigerators where the average relative humidity was 99 per cent and the average temperature 60° F. They also germinated readily on moist slides in a cellar where the temperature ranged from 60° to 70°. Sporidia which had been dried and exposed for 26 hours (*14*) to temperatures of 58° to 62° and 60° to 62° and relative humidities of 80 to 85 per cent and of 74 to 84 per cent, respectively, were able to germinate, but no tests were made to determine whether they could infect pines.

In 1922 pines in situ (*7*) became infected within and without iceless refrigerators at high relative humidities and at rather low temperatures. In 1921 (*17*) potted pines became infected at an average relative humidity of 94 per cent and an average temperature of 65° F. They also became infected when the average relative humidity was 88 per cent. In 1922 some potted pines became infected in every experiment. The relative humidities ranged from 74 to 99 per cent and the temperatures from 51° to 72°. The relative humidity was above 90 per cent most of the time, and it was commonly above 95 per cent. From these results some, but not the extreme, ranges of temperatures and the relative humidities which permit infection can be ascertained. The temperatures and relative humidities which prevent infection are not yet known.

RELATIVE SUSCEPTIBILITY OF 1, 2, AND 3 YEAR OLD NEEDLES OF PINUS STROBUS

Clinton (*2*) and the writers (*7, 17*) stated that both the first and second year needles of *Pinus strobus* may become infected, but they gave no data on the relative susceptibility of needles of different ages. In an attempt to get some definite information on this point in 1922, 60 inoculations were made on needles of known ages on older trees. Two per cent of these inoculations were made on 1920 needles, 18 per cent on 1921 needles, and 80 per cent on 1922 needles. None of the inoculations on 1920 needles, 18 per cent of those on the 1921 needles, and 19 per cent of those on the 1922 needles developed leaf spots. None of the inoculations on 1920 needles, 9 per cent of those on 1921 needles, and 8 per cent of those on the 1922 needles resulted in twig cankers. These results agree with the statement previously made that 1921 and 1922 needles seemed of approximately equal susceptibility (*7*).

One hundred and twenty-seven other cankers resulting from inoculations made on the pines in situ in 1922, were examined in October, 1925, in order to ascertain the age of wood upon which they occurred. Ninety-five per cent of these cankers were on 1922 wood and 5 per cent on 1921 wood. These results confirm the above statement that both 1 and 2 year old needles are susceptible. They do not, however, give any information on the relative susceptibility of 1921 and 1922 needles, for no record was kept of the relative percentages of the 1921 and 1922 needles originally inoculated. The most that can be said is that no indication was encountered that 2-year-old needles of *Pinus strobus* are more liable to infection than 1-year-old needles.

External symptoms as well as sectioning indicate that the 1920 as well as the 1921 needles of potted seedlings inoculated in 1921 were susceptible to infection. The 1921 and 1922 needles of potted seedlings inoculated in 1922 were also susceptible. However, no 1922 seedling became definitely infected. The fact that both 1-year-old and 2-year-old needles are susceptible increases the chances of infection.

Approximately 40 per cent of the 1922 inoculations of branches of pines in situ which had spotted needles in June, 1923, developed no further symptoms of infection between that date and October, 1925. None of the spotted needles were sectioned because a maximum number of twig infections were desired, and it is entirely possible that some of the needle spots were caused by something besides blister rust infections. Approximately one-tenth of the potted seedlings inoculated in 1921 whose needles became spotted developed no further symptoms of rust infection. Sections made by the method described by Colley (*3*) showed that the needles of one of these seedlings were definitely infected. Also about one-quarter of the potted seedlings inoculated in 1922 which developed leaf spots showed no further evidence of infection. Spaulding (*11*) stated that the relative susceptibility of the different white pines to *Cronartium ribicola* seems to be associated with the length of time the needles persist and that the needles of *Pinus strobus* persist for two to three years. He concluded, "with *P. strobus* there is a chance that the older infected leaves may drop before the fungus reaches the bark of the twig." Spaulding's

theory probably accounts for the presence of rust symptoms in needles and their subsequent absence in twigs in some of the writers' experiments, but as spots resembling those caused by blister rust are sometimes present in needles which are not infected other causes may account for some of them. For this reason it is necessary to section and stain spotted pine needles in order to be sure that the needle spots are white pine blister rust infections.

On the other hand, a large number of infections occurred on the inoculated twigs of the pines in situ without needle spots having been observed on the dates of inspection. About half of the potted seedlings which eventually showed symptoms of blister rust developed no leaf spots which were visible at the times of inspection.

RELATIVE VIRULENCE OF SPORIDIA FROM DIFFERENT RIBES HOSTS

It has been stated that by September, 1924, none of the sporidia from *Ribes cynosbati* had produced external symptoms of infection on the trees in situ inoculated with them in 1922, but that sporidia from *R. odoratum* and those from *R. nigrum* from two localities had caused infections with typical external symptoms (7). In 1925 it was found that sporidia from *R. cynosbati* had also caused some infections on the inoculated trees. Inoculations with sporidia from different hosts were not made simultaneously. For this reason and because so many inoculated twigs and branches were lost in the logging operations mentioned above, it is impossible to compare directly the infection precentages of the sporidia from the different hosts. The indications are, however, that the infection percentages were influenced mainly by factors other than the hosts upon which the sporidia were produced.

Sporidia from *Ribes nigrum* only were used in the 1921 inoculations. Sporidia from *R. cynosbati*, from *R. odoratum*, and from *R. nigrum* collected in two localities all caused some infection on potted pines inoculated in 1922. The inoculations with sporidia from the different Ribes hosts were not made simultaneously, but the results are presented in Table 5 for what they are worth. Comparisons of the infection percentages seem to indicate that sporidia from *R. cynosbati* were less virulent than those from the other two hosts but comparisons of the percentages of healthy seedlings remaining in October, 1925, seem to indicate that sporidia from all three hosts were of approximately equal virulence.

TABLE 5.—*Relative virulence of sporidia of Cronartium ribicola from different species of Ribes*

Source of sporidia	Number of seedlings inoculated	Per cent infected [a]	Per cent healthy [a]
Telia from Ribes cynosbati [b]	27	19	30
Telia from Ribes odoratum [b]	24	33	29
Telia from Ribes nigrum [b] (North Conway, N. H.)	51	39	28
Telia from Ribes nigrum [b] (Twin Mountain, N. H.)	83	30	34
Do.[c]	45	31	29

[a] These percentage figures do not total 100 because a great many of the seedlings died before the presence or absence of infection had been determined.

[b] Teliospores pregerminated when suspended over seedlings and placed in iceless refrigerator.

[c] Telia ungerminated when suspended over seedlings and placed in iceless refrigerator.

This would mean that if *Ribes odoratum* and *R. cynosbati* produced as many sporidia as *R. nigrum* (*15*), bushes of those species would be just as dangerous to near-by pines as those of *R. nigrum*. General field experience corroborates this suggestion. There is, however, some indication which needs more definite proof that sporidia from *R. nigrum* are more tolerant of drying than those from other species (*14*).

TIME ELAPSING BETWEEN INOCULATION AND PRODUCTION OF PYCNIA AND AECIA

In October, 1925, 140 cankers on pines in situ inoculated in 1922 were examined for evidences of the production of pycnia and aecia. Only 14, or 10 per cent, of these had produced pycnia and only 4, or 3 per cent, had produced aecia. This makes the period elapsing between infection and the first production of aecia approximately three years. The first pycnia were produced at some earlier date. For most of the cankers the period between infection and the production of pycnia and aecia will be more than three years. Most of the infected potted seedlings died without producing either pycnia or aecia.

Spaulding (*9*) summarized the known data on the incubation period of *Cronartium ribicola* on pines. Artificially infected seedlings of *Pinus strobus* produced pycnia 5 to 6 months (*2*), 10 months (*6*, *16*), and 13 months (*8*) after inoculation. The incubation periods for blister rust in these seedlings, most of which were inoculated in greenhouses, were naturally shorter than those in the writers' out-of-doors experiments. Tubeuf (*16*) observed aecia 32 months after inoculation, which is approximately the same length of time as that observed by the writers.

SUMMARY

Sporidia from teliospores which had been allowed to germinate in moist chambers for six hours before being suspended over potted seedlings in an iceless refrigerator caused on the whole neither more nor quicker infection than sporidia from teliospores which were ungerminated when placed in the iceless refrigerator.

Infections occurred at rather high relative humidities and at medium temperatures. The extreme temperature and relative humidity ranges suitable for infection have not been determined.

Sporidia from *Ribes cynosbati*, *R. odoratum*, and *R. nigrum* were able to infect both the potted seedlings and the pines in situ. Sporidia from other Ribes were not tested.

External symptons as well as sectioning showed that both 1 and 2 year old needles of *Pinus strobus* are susceptible to sporidia of *Cronartium ribicola*. So far as ascertained, the younger and older needles are approximately equally susceptible. Cankers developed on wood which was formed in the same season in which inoculations were made, as well as on wood which was formed in the preceding season.

External evidences of infection confirmed by sectioning were present in the needles three months after inoculation.

Potted seedlings of the current year in certain experiments, as well as older ones, became infected.

Some of the infected potted seedlings had died one year after inoculation. Mortality was higher in inoculated potted seedlings which were known to have been infected than in inoculated ones on which no infection was visible.

Only a small percentage of the infections had produced either pycnia or aecia three years after inoculation.

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A MORPHOLOGIC AND BIOMETRIC COMPARISON OF CRONARTIUM RIBICOLA AND CRONARTIUM OCCIDENTALE IN THE AECIAL STAGE [1]

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INTRODUCTION

Ever since the piñon blister rust, *Cronartium occidentale* Hedgcock, Bethel, and Hunt (*5*),[3] was discovered to be widespread in certain sections of the Western States, the question has been raised as to whether it was really distinct from the white pine blister rust, *Cronartium ribicola* Fischer. The former is relatively innocuous, attacking—so far as is known—only the nut pines; but the latter is a particularly destructive parasite infecting the valuable 5-needled pines. The uredinial and telial stages of both forms occur on members of the genus Ribes. A recent study (*3*) has shown that the urediniospores of the two species are very close together biometrically but fairly consistently different, and that the size and shape of the spores are reasonably reliable criteria for diagnosis. The purpose of the present paper is to submit the evidence of morphologic and biometric differences in the aecial stages. These differences appear to be both significant and easily recognized.

MATERIAL

The chief source of material was the office collection. In addition, specimens which had been specially preserved in formalin-alcohol (6 c. c. full strength [37 per cent] commercial formaldehyde to 94 c. c. of 70 per cent alcohol) and specimens fresh from the field were used. Slides were made from these preserved and fresh specimens; and slides which had been prepared in previous work on the morphology and cytology of *Cronartium ribicola* (*1*) were carefully restudied.

Herbarium material only was selected for the biometric study of the aeciospores and peridia. For each species apparently representative specimens, with as wide a locality and time of collection range as possible, were chosen. Most of the *Cronartium ribicola* material was on the one host *Pinus strobus* L., although there were three specimens on *P. monticola*, but the *C. occidentale* selections were about equally divided between *P. edulis* Engelm. and *P. monophylla* Torr. and Frem. The collection data are given in full in Table 1.

[1] Received for publication Nov. 17, 1926; issued May, 1927.
[2] The writers wish to acknowledge the help rendered by their colleagues, without which it would have been very difficult indeed to carry on the study reported herein. They are also deeply indebted to the members of the Offices of Forest Pathology and Blister Rust Control for specimens, for assistance in the tabulation and analyses of the measurement data, and for much helpful criticism.
[3] Reference is made by number (italic) to "Literature cited," p. 531.

TABLE 1.—*Biometric data on aeciospores of Cronartium ribicola and C. occidentale, based on 100 spores from each specimen*

Host	Locality	Date collected	Length		Width		Mean wall thickness	Ratio: Mean length, mean width
			Mean	Standard deviation	Mean	Standard deviation		
Cronartium ribicola:			μ		μ		μ	
Pinus strobus.	Dunraven, N. Y.	May 20, 1915	24.4	2.3	17.9	1.5	3.36	1.36
Do.	East Randolph, Vt.	May 18, 1916	25.1	2.0	18.1	1.5	3.04	1.38
Do.	Woodstock, Vt.	do.	24.0	2.0	17.7	1.4	2.98	1.35
Do.	Kittery Point, Me.	May —, 1917	24.8	2.0	17.9	1.5	2.82	1.38
Do.	do.	1917	25.2	2.2	19.1	1.4	3.69	1.31
Do.	do.	May 18, 1917	24.8	2.9	18.5	1.9	.....	1.34
Do.	Exeter, N. H.	May 24, 1917	25.6	1.8	19.7	1.3	3.69	1.29
Do.	Stratham, N. H.	May —, 1917	25.9	1.9	19.4	1.4	3.34	1.33
Do.	Marlow, N. H.	June 14, 1917	24.3	2.9	19.1	1.8	4.18	1.27
Do.	Gloversville, N. Y.	Sept. —, 1917	23.9	2.4	14.9	1.9	.....	1.60
Do.	Kingston, Mass.	Mar. 22, 1918	23.3	2.4	17.7	1.5	3.10	1.32
Do.	Brunswick, Me.	Apr. 30, 1918	24.0	2.0	18.7	1.4	3.20	1.28
Do.	Halifax, Mass.	May 13, 1918	22.6	1.8	18.2	1.4	3.00	1.24
Do.	Maine	May —, 1919	24.1	1.9	19.0	1.5	3.24	1.27
Do.	Block Island, R. I.	May —, 1920	24.0	2.6	17.7	1.8	.....	1.35
Do.	Portland, Me.	June —, 1920	24.0	2.0	18.0	2.9	.....	1.33
Do.	North Conway, N. H.	do.	24.9	4.1	16.7	2.1	.....	1.49
Do.	Topsfield, Mass.	Mar. —, 1921	22.3	2.1	18.1	2.0	.....	1.23
Do.	do.	Mar. 27, 1921	24.0	2.2	18.7	1.5	3.17	1.28
Do.	Block Island, R. I.	Apr. 6, 1921	22.0	1.9	17.2	1.5	2.81	1.27
Do.	South Deerfield, N. H.	May —, 1922	23.7	2.2	18.3	1.8	3.23	1.29
Do.	Vancouver, B. C.	Mar. —, 1922	22.4	2.8	16.8	1.6	2.97	1.33
Pinus monticola.	North Vancouver, B. C.	June —, 1922	25.9	2.2	19.6	1.6	4.29	1.32
Do.	do.	June 7, 1922	26.3	2.0	20.6	1.6	[1] 4.50	1.27
Do.	Vancouver, B. C.	June 9, 1922	24.3	2.0	19.2	1.6	[1] 4.17	1.26
Constants computed from specimen means.	.....	.....	24.2	1.11	18.3	1.14	3.41	[2] 1.32
Cronartium occidentale:								
Pinus edulis.	Bayfield, Colo.	May 19, 1918	27.2	2.6	18.9	1.8	4.01	1.43
Do.	Trimble Hot Springs, Colo.	June 23, 1918	25.7	2.1	18.6	2.2	3.48	1.36
Do.	Mancos, Colo.	June 28, 1918	26.5	2.3	19.3	2.0	3.51	1.37
Do.	do.	June 29, 1918	27.4	3.0	20.0	2.1	3.71	1.37
Do.	Mesa Verde National Park, Colo.	July —, 1918	25.8	2.6	19.0	2.4	.....	1.35
Do.	Glenwood Springs, Colo.	May 28, 1919	25.7	2.4	18.8	1.4	3.62	1.36
Do.	Bayfield, Colo.	Oct. 15, 1920	28.8	3.3	19.4	2.4	4.29	1.48
Do.	Mancos, Colo.	Oct. 16, 1920	23.6	2.3	18.5	1.8	3.64	1.27
Pinus monophylla.	Minden, Nev.	May —, 1920	26.7	2.7	16.8	2.7	.....	1.59
Do.	do.	May 20, 1920	28.1	3.3	20.9	2.4	4.39	1.34
Do.	do.	May 22, 1920	28.5	2.7	20.6	1.6	4.75	1.38
Do.	do.	May 30, 1920	27.1	2.9	18.9	1.6	3.97	1.43
Do.	Bridgeport, Calif.	May 31, 1920	27.8	3.0	18.3	2.0	3.31	1.51
Do.	Walker Canyon, Calif.	do.	27.1	2.5	18.0	1.8	3.78	1.50
Do.	Minden, Nev.	June —, 1920	27.1	2.9	18.2	2.2	.....	1.49
Do.	do.	Aug. —, 1920	26.3	2.6	18.9	2.6	.....	1.39
Do.	Bethel Collection.	Sept. —, 1920	26.4	2.8	19.0	2.2	.....	1.39
Do.	Bridgeport, Calif.	Sept. 12, 1920	28.1	2.5	20.5	2.5	4.03	1.37
Do.	Sweetwater, Nev.	Sept. 14, 1920	27.0	2.9	19.6	2.2	3.40	1.37
Do.	Carter's Station, Nev.	June —, 1921	25.3	3.4	18.1	2.3	.....	1.39
Do.	Bridgeport, Calif.	May 31, 1920	27.5	3.7	.....	.....	.....	.....
Constants computed from specimen means.	.....	.....	26.8	1.18	19.0	.95	3.85	[2] 1.41

[1] Based on 200 spores.

[2] Geometric means of the above ratios.

METHODS

SECTIONING, STAINING, AND MOUNTING

The killing, sectioning, staining, and mounting methods described in the earlier paper on *Cronartium ribicola* (*1*) were followed in preparing slides for the study of the mycelium in the bark and wood, and the young aecia. The mature peridia were sectioned for the measurement study and for observations on comparative morphology either with hand razors or on an ether freezing microtome. The use of two sections of a Lima bean for holding the peridia, suggested by N. A. Cobb, made the sectioning process comparatively easy in spite of the brittle character of the objects. The hardness of the Lima bean was adjusted by soaking and redrying until it was just right for the job.

Aeciospores were shaken out of the aecia (not scraped out) or picked up on a knife blade from the bottom of the packet containing the specimen, and mounted in the same glycerin and glycerin-jelly media that were used in the study of the urediniospores (*3*).

Fragments and sections of the peridia were mounted in the same way.

It will be noted that the method of getting the aeciospores into the mounts assured both mature spores and a fairly good sampling of the spores from any particular specimen. Each mount made by shaking the spores out of the aecia into the mounting media presumably contained spores from several aecia; and each mount made from the mass of spores which were lying loose in the herbarium packet presumably contained a mixture of spores from all the opened aecia on the specimen.

MEASURING

The great majority of the spores were measured by means of a projection apparatus (*2*). The images of the spores, at a magnification of 1,000 diameters, were thrown on a white field; and those images which fell within a 4-inch circle in the center of this field were measured to the nearest millimeter with a white-face millimeter scale.

In all of the measurement work the mount was moved across the field of vision systematically by means of a mechanical stage. One hundred spores were measured from each of the specimens of each species. The thickness of the thickest visible part of the side spore wall was measured on 100 spores of each of 19 specimens of *Cronartium ribicola* and 14 specimens of *C. occidentale*.

The tubercles on the aeciospores were measured with a filar micrometer.

Measurements of the peridial cells, also made with a filar micrometer, were limited to the cells of the outer layer of the peridium. The walls measured were the outer walls of these same cells. The measurement results are presented in Tables 1 to 6.

TABLE 2.—*Biometric data on the tubercles on the aeciospores of Cronartium ribicola and C. occidentale, based on 100 tubercles from each specimen*

Host	Locality	Date collected	Length		Width		Ratio: Mean length, mean width
			Mean	Standard deviation	Mean	Standard deviation	
			μ		μ		
Cronartium ribicola:							
Pinus strobus	Dunraven, N. Y	May 20, 1915	1.64	0.35	1.16	0.24	1.41
Do	Exeter, N. H	May 24, 1917	1.56	.25	1.15	.22	1.35
Do	Kingston, Mass	Mar. 22, 1918	1.52	.28	1.10	.22	1.38
Do	Block Island, R. I	May —, 1920	1.47	.28	1.06	.18	1.38
Do	Topsfield, Mass	Mar. —, 1921	1.39	.26	1.04	.17	1.33
Pinus monticola	North Vancouver, B. C.	June —, 1922	1.51	.32	1.08	.21	1.39
Do	do	June 7, 1922	1.53	.45	1.11	.21	1.37
Constants computed from specimen means.			1.52	.071	1.10	.041	[1] 1.37
Cronartium occidentale:							
Pinus edulis	Bayfield, Colo	May 19, 1918	2.26	.53	1.31	.24	1.72
Do	Mancos, Colo	June 29, 1918	2.30	.69	1.36	.29	1.69
Pinus monophylla	Minden, Nev	May 22, 1920	2.21	.54	1.33	.26	1.66
Do	do	May —, 1920	2.26	.85	1.34	.29	1.68
Do	Carters Station, Nev.	June —, 1921	2.40	.62	1.32	.27	1.81
Constants computed from specimen means.			2.29	.063	1.33	.017	[1] 1.71

[1] Geometric means of the above ratios.

TABLE 3.—*Biometric data on the outer layer of peridial cells in the aecia of Cronartium ribicola and C. occidentale, based on 100 cells from each specimen*

Host	Locality	Date collected	Length		Width		Wall		Ratio: Mean length, mean width
			Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	
			μ		μ		μ		
Cronartium ribicola:									
Pinus strobus			42.7	6.0	31.5	7.3	6.16	1.24	1.35
Do	Kittery Point, Me.	May —, 1917	42.8	6.6	29.8	5.1	7.25	1.64	1.43
Do			44.1	7.2	29.4	4.5	7.45	1.64	1.50
Do	Kittery Point, Me.	May —, 1917	38.8	8.4	26.5	4.5	6.19	1.17	1.46
Do	do	do	42.8	6.4	28.7	4.5	6.82	1.10	1.49
Pinus monticola.	North Vancouver, B. C.	June 7, 1922	38.4	6.6	27.2	5.9	6.52	.....	1.41
Do	Vancouver, B. C.	June 9, 1922	43.7	6.7	30.4	5.7	7.74	.....	1.43
Do	North Vancouver, B. C.	June, —, 1922	38.2	6.9	24.3	5.1	9.12	.....	1.57
Constants computed from specimen means.			41.4	2.35	28.5	2.19	7.16	.919	[1] 1.45
Cronartium occidentale:									
Pinus edulis	Bayfield, Colo	May 19, 1918	27.0	3.4	20.6	3.6	4.48	.81	1.31
Do	do	do	25.5	3.5	18.0	3.7	4.14	.90	1.41
Pinus monophylla.	Bridgeport, Calif.	May 31, 1920	28.7	4.9	18.7	2.9	5.00	.88	1.53
Do	do	do	27.8	3.7	18.6	3.5	4.95	1.02	1.49
Do	do	do	28.1	4.8	19.6	2.8	4.84	.82	1.43
Constants computed from specimen means.			27.4	1.10	19.1	.91	4.68	.326	[1] 1.43

[1] Geometric means of the above ratios.

TABLE 4.—*Biometric data on the "concomitant" cells in the aecia of Cronartium occidentale, based on 100 cells from each specimen*

Host	Locality	Date collected	Length		Width		Mean wall thickness	Ratio: Mean length mean width
			Mean	Standard deviation	Mean	Standard deviation		
			μ		μ		μ	
Pinus edulis	Bayfield, Colo	June 19, 1918	21.8	3.0	17.2	2.5	4.64	1.26
Pinus monophylla	Bridgeport, Calif.	May 31, 1920	21.9	2.3	18.5	2.1	4.92	1.18
Do	do	do	21.5	2.4	18.0	2.4	4.35	1.19
Do	do	do	22.4	2.5	17.8	2.0	4.56	1.25
Do	Minden, Nev	June —, 1920	22.0	2.5	18.0	2.4	4.54	1.22
Constants computed from specimen means			21.9	.29	17.9	.42	4.60	[1] 1.22

[1] Geometric mean of above ratios.

TABLE 5.—*Biometric constants for Cronartium ribicola and C. occidentale, computed from the populations of specimen means*

Dimensions measured	Cronartium ribicola					Cronartium occidentale					Difference between the two fungi			
	Number of specimens	Mean	Probable error of the mean	Probable deviation of a single specimen	Coefficient of variability	Number of specimens	Mean	Probable error of the mean	Probable deviation of a single specimen	Coefficient of variability	Difference between means	Probable error of the difference	Ratio: Difference, error	Diagnostic division point
Aeciospores:														
Length	25	24.2	0.15	0.76	4.6	21	26.8	0.18	0.81	4.4	2.6	0.23	11.3	25.5
Width	25	18.3	.16	.79	6.2	20	19.0	.15	.65	5.0	.7	.21	3.8	18.7
Wall	19	3.41	.081	.353	14.9	14	3.85	.074	.279	10.4	.440	.109	4.0	3.66
Length / Width	25	[1] 1.32	.010	.053	5.8	20	[1] 1.41	.011	.050	5.1	.09	.015	5.3	1.365
Aeciospore tubercles:														
Length	7	1.52	.019	.051	4.7	5	2.29	.022	.048	2.8	.77	.029	26.6	1.92
Width	7	1.10	.011	.029	3.7	5	1.33	.006	.013	1.3	.23	.012	19.3	1.26
Peridial cells:														
Length	8	41.4	60	1.70	5.7	5	27.4	.37	.83	4.0	14.0	.70	20.0	31.9
Width	8	28.5	.56	1.58	7.7	5	19.1	.31	.68	4.8	9.4	.64	14.7	21.9
Wall	8	7.16	.233	.661	12.8	5	4.68	.110	.246	7.0	2.48	.257	9.6	5.33

[1] Geometric means.

TABLE 6.—*Summary of measurements of aeciospores of Cronartium ribicola and C. occidentale from different hosts*

Host	Number of specimens	Length	Width	Wall thickness
		μ	μ	μ
Cronartium ribicola:				
On Pinus strobus	22	24.0±0.15	18.1±0.15	[1] 3.24±0.06
On Pinus monticola	3	[2] 25.5± .41	[2] 19.8± .41	[2] 4.32± .14
Differences		1.5± .44	1.7± .44	1.08± .15
Cronartium occidentale:				
On Pinus monophylla	12	27.2± .16	19.0	3.95
On Pinus edulis	8	26.3± .37	19.1	3.61
Differences		.9± .40	.1	.34

[1] Based on 16 specimens only.
[2] Probable errors for these 3-specimen means are not computed from the population of 3 specimens. They are assumed to be the same percentage of the means as would be expected for 3-specimen means from *P. strobus*.

GENERAL OBSERVATIONS

There are certain differences in the habit of *Cronartium ribicola* and *C. occidentale* which are clearly evident to anyone who has had the opportunity to examine representative specimens or to observe the fungi in the field. For example, the pycnia of *C. ribicola* on *Pinus strobus* (*1, pl. 48, fig. B; 7, pl. II, fig. 2*), though occasionally confluent, are usually discrete and conspicuous; whereas the pycnia of *C. occidentale* on *P. monophylla* are made up of broad confluent groups of spore-bearing cells hidden beneath the overlying grayish outer bark.

The typical aecia of *Cronartium ribicola*, as they appear in the smooth soft bark of *Pinus strobus* (*1, pl. 48, fig. B; 5, pl. 55*), are usually separate sori, although occasionally they run together. The peridia, puffed out by the growing aeciospore chains, protrude through the bark—conspicuous bits of evidence that the host is infected. The aecia of *C. occidentale*, particularly on *P. edulis*, are broad confluent spore-producing layers which are more or less completely hidden under the hard bark—the spores massed at a crack in the bark being the only indication of infection. The area of the spore-bearing surface can not be determined until the overlying bark is removed. On young twigs of *P. monophylla*, however, the aecia are small, the peridia protrude, and the general appearance of the infected branch reminds one very strongly of branches of *P. strobus* infected with *C. ribicola*.

Hedgcock, Bethel, and Hunt (*5, p. 414*) are of the opinion that the difference in morphology is not due to the physical difference in the bark of the hosts. In the case of *Cronartium ribicola*, at least, the aecium which develops under the horny, resin-infiltrated bark of the canker area does not expand radially or in any way take on the character of the aecium of *C. occidentale* as it is commonly found under the hard bark of *Pinus edulis*. Aecia on the roots of *P. strobus* appear to be normal in size, shape, and structure (*1, p. 648*). There seems to be no way of ascertaining what form *C. ribicola* would take in the bark of *P. edulis*, or what form *C. occidentale* would take in the bark of *P. strobus*, unless it proves possible to grow the fungi on the hosts indicated. In the face of lack of evidence to the contrary, one is forced to conclude that the difference in habit of the aecia is real and specific. One must also conclude, however, that the type of aecium on the young branches of *P. monophylla* is totally unlike the confluent type on *P. edulis*. The former might be taken for *C. ribicola*, but the latter never. This fact and the data in Table 6 suggest that there may be a varietal difference between *C. occidentale* on *P. edulis* and *C. occidentale* on *P. monophylla*. The question of gradations between the type on the young branches of *P. monophylla* and the type on *P. edulis* must be passed over for the present.

MORPHOLOGIC AND BIOMETRIC COMPARISON

MYCELIAL CHARACTERS

The difference in the character of the bark of *Pinus strobus* and *P. edulis* naturally produces a difference in the appearance of sections of infected tissue. The hyphae and haustoria of the two rusts vary little in habit. The mean diameter of the hyphae in the wood for

both rust species, based on a total of 100 measurements made on six different specimens of each, is 4.9 μ. The walls of the same set of hyphae have a mean thickness of approximately 1 micron. Comparative measurements on the haustoria of the two forms—made on the largest haustoria, and therefore definitely selective—confirmed an impression gained from examination of hundreds of slides that the haustoria of *Cronartium ribicola* are longer than those of *C. occidentale*. The means of 100 measurements (*C. ribicola*, five specimens on *P. strobus; C. occidentale*, four specimens on *P. monophylla*) are as follows:

Haustoria of *C. ribicola*..... 29.2μ long by 5.4μ in diameter, with a wall 1.1μ thick.

Haustoria of *C. occidentale*..... 25.8μ long by 5.4μ in diameter, with a wall 1.1μ thick.

It is evident that difference in size, as far as these few specimens are concerned, exists only in the length of the haustoria. The difference is six and five-tenths times its probable error. The number of specimens sampled was so small, however, that positive conclusions are not justified. The character of the haustoria may be influenced by the difference in host.

COMPARATIVE MORPHOLOGY OF THE AECIOSPORES OF THE TWO SPECIES

The aeciospores of the two species appear to be very similar under medium power lenses. Under higher powers they are distinctly different. Both species produce characteristic Peridermium spores—obovoid to ellipsoid in shape, with a wall partly smooth and partly coarsely verrucose, as illustrated in Figure 1. Three points of difference in morphology stand out fairly clearly when the spores are compared, namely: The aeciospores of *C. ribicola* (fig. 1, A to G) are slightly smaller than those of *C. occidentale* (fig. 1, H to N); their outline is somewhat more regular; and their tubercles are more regular in shape and more evenly distributed. These differences have been taken into consideration in drawing the figures lettered O and P in Figure 1, which represent mean aeciospores of *C. ribicola* and *C. occidentale*, respectively, constructed on the basis of the mean size figures given in Tables 1 and 2.

One other difference which can not be illustrated in a drawing is a difference in light refraction at the edge of the spore. The wall and tubercles of *Cronartium occidentale* are more refractive than those of *C. ribicola*; and the difference is great enough to enable one familiar with the spores to pick out those of one species from those of the other when both forms are mounted under one cover. The greater refraction in *C. occidentale* appears to be due to the more irregular shape and distribution of the tubercles.

BIOMETRIC COMPARISON OF THE AECIOSPORES

The values given in this and the following sections will be found to differ only slightly from those published in the preliminary report (*4*) on part of the biometric study. The data are summarized in Table 1 and represented graphically in Figure 2. The mean wall thickness refers to the mean of measurements made for each spore at the thickest visible part of the side wall.

The aeciospores reach almost mature size soon after the division of the aeciospore initial into the aeciospore and intercalary cell. For instance, if the spores be numbered from the base of the aecio-

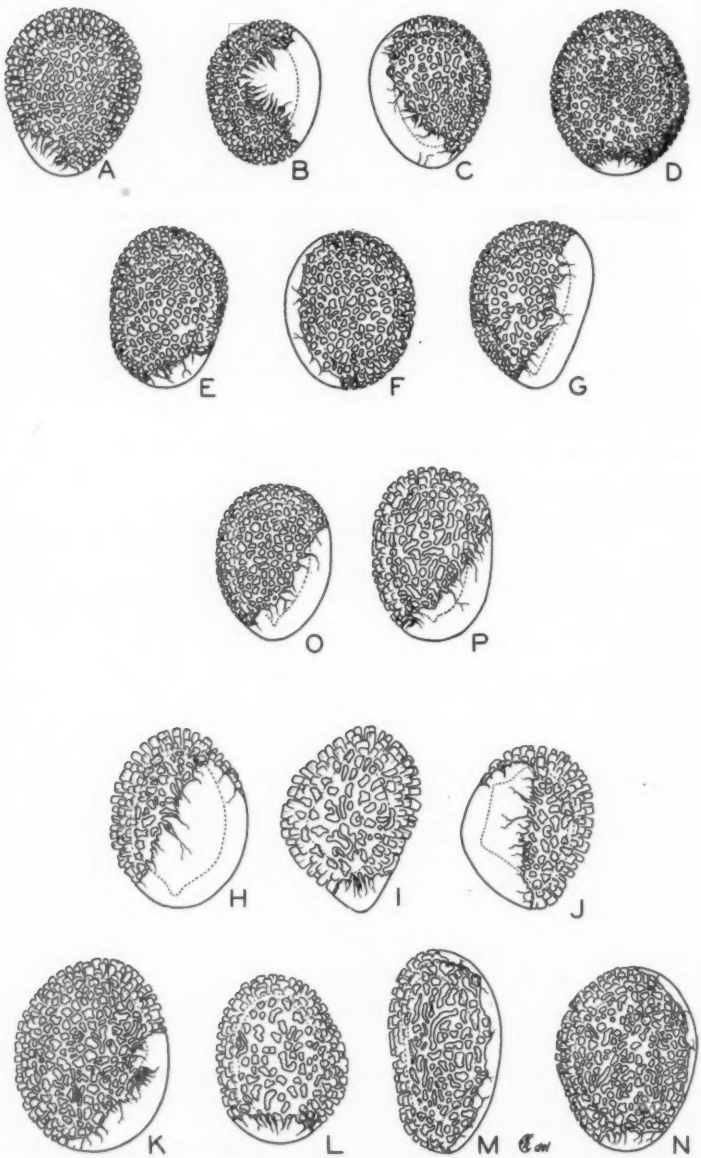

FIG. 1.—A to G, aeciospores of *Cronartium ribicola*, × 900; H to N, aeciospores of *C. occidentale*, × 960; O and P, mean aeciospores of *C. ribicola* and *C. occidentale*, respectively, based on the mean size and shape data given in Tables 1 and 2

spore chain, No. 1 being the youngest, it will be found that the spores in No. 2 position are nearly full size in length, and that those in No. 3 and No. 4 positions are very close to the mean size figures of Table 1, though the walls are still decidedly thinner than in fully matured spores. The following data, from measurements made with a filar

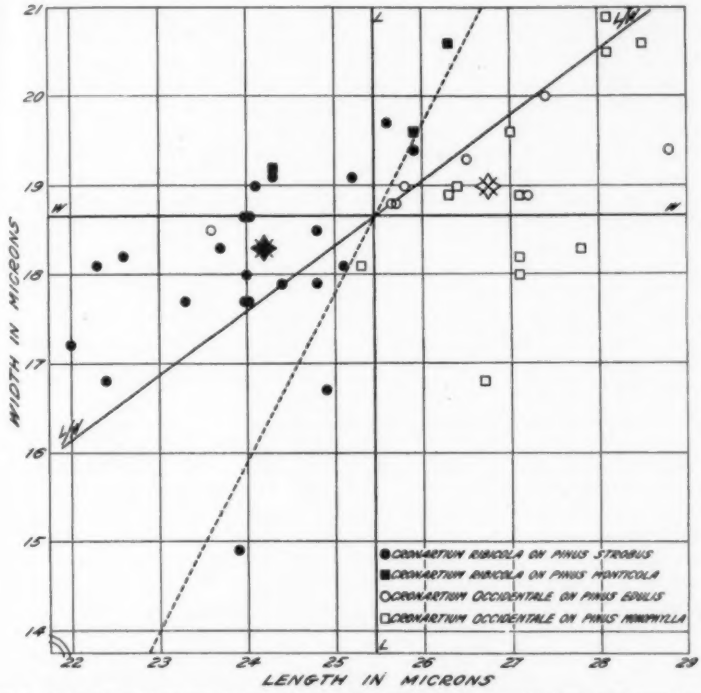

FIG. 2.—*Cronartium ribicola* and *C. occidentale*. Graphic methods for separating two species according to length and width of spores. The points are based on 100-spore means, and each represents a different specimen. The stars indicate the means for the two species. The solid lines are the most probable diagnostic separation lines determined by computation; the line marked W is for width, the line L is for length, and the line marked L/W is for the ratio of length to width. The broken line is located empirically as being apparently the best straight line for separating the two species

micrometer on stained sections mounted in balsam, support the conclusion:

Cronartium ribicola (two specimens, three slides from each):
Second spore in chain.
Means from 26 spores, 23.5×11.7μ, wall 1.9μ.
Third spore in chain.
Means from 20 spores, 24.1×15.0μ, wall 1.9μ.
Fourth spore in chain.
Means from 4 spores, 23.6×13.4μ, wall 1.9μ.
Cronartium occidentale (three specimens, one slide from each):
Second spore in chain.
Means from 26 spores, 26.7×16.6μ, wall 2.7μ.
Third spore in chain.
Means from 20 spores, 28.3×17.3μ, wall 2.8μ.
Fourth spore in chain.
Means from 4 spores, 27.3×17.1μ, wall 2.7μ.

Measurements made on stained spores mounted in balsam are, of course, not directly comparable with the measurement figures in Table 1; and the number of measurements reported is small; but the means are at least indicative. The relatively larger size and thicker wall of the aeciospores of *Cronartium occidentale* are apparently characteristic even before the spores are mature.

Hedgcock, Bethel, and Hunt (*5*) have already called attention to the size of the tubercles on the aeciospores of *Cronartium occidentale* in their description of the species. Morphologic differences in such minute things as tubercles are easily overlooked when one is studying rust spores in a routine way under the medium power of the microscope; yet the recognition of these differences is essential to any true diagnostic description. Data on the size and shape of the aeciospore tubercles are given in Table 2. Length and width in the case of the tubercles mean the long and short dimension, as nearly as they can be measured, of the end views of the tubercles as they appear in Figure 1. The means in the summaries of Table 2 were obtained from measurements of 500 tubercles for *C. occidentale* and 700 for *C. ribicola*, approximately 5 of the most clearly visible tubercles having been measured on each of 20 spores in the case of each of the specimens. The greater length and greater irregularity in shape and distribution of the tubercles in *C. occidentale* is clearly evident in the spores shown in Figure 1.

COMPARATIVE MORPHOLOGY OF THE PERIDA OF THE TWO SPECIES

General observations have indicated a decided difference in the peridia of the two forms. The peridium of *Cronartium ribicola* has been described as thick and persistent in contrast to the inconspicuous, "thin, evanescent" (*5, p. 414*) peridium of *C. occidentale*. Colley's drawings of a section of the peridium of *C. ribicola* (*1, pl. 56, fig. B*) show it as three to four cells thick. Vertical sections through the aecium (*1, pl. 50, fig. B*) show that this multilayered cover extends all over the aecium, and that before the rupture of the bark by the force of the growth of the aeciospore chains the peridium forms a very definite layer between the tips of the young spore chains and the overlying bark and "buffer" tissue. The peridium of *C. occidentale*, whether it remains closely pressed against the overlying bark tissue of *Pinus edulis*, or whether it protrudes through the bark as in the case of infections on young branches of *P. monophylla*, generally appears to be but one cell in thickness. Occasionally a few cells are found in the position of a second, or inner, layer.

The thickness of the cell wall decreases in each layer of cells in the peridium of *Cronartium ribicola* (fig. 3, A); and as a rule the outer wall is thicker than the inner wall of any given cell. The wall thickness remains practically constant for any given cell in *C. occidentale*. Under proper illumination the thick walls are beautifully striated (fig. 3, A and B). The cells retain some of their contents even after the peridia become quite dry. Only rarely are the inner walls of the outer layer of cells marked with tubercles in *C. occidentale;* whereas all the cells are sculptured in the case of *C. ribicola*. The sections shown in the drawings are typical of a large number examined, taken from numerous specimens and localities.

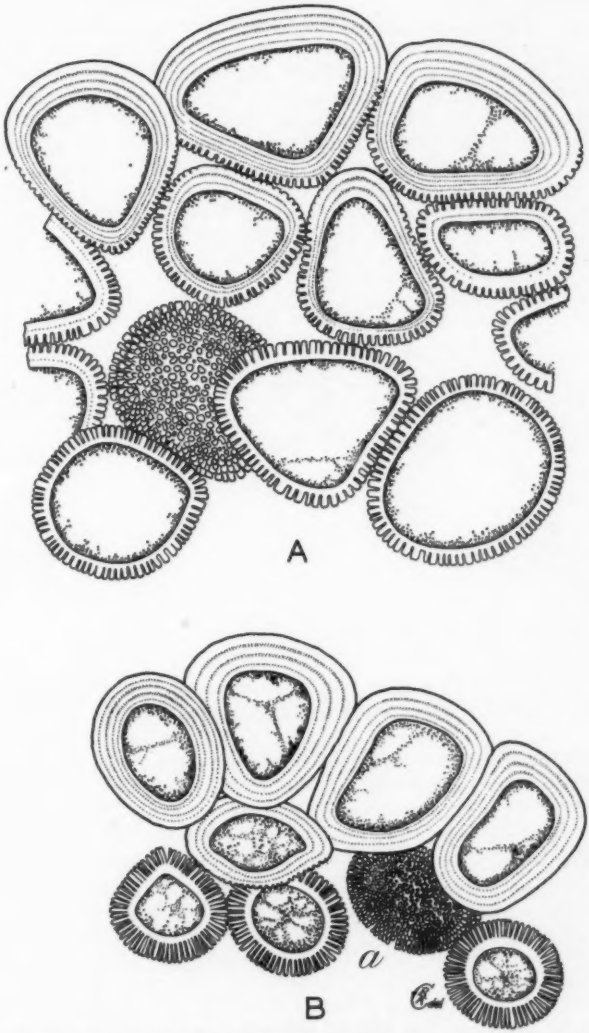

FIG. 3.—A detailed sectional view of small segment of roof of peridium: A, *Cronartium ribicola* on *Pinus strobus;* B, *C. occidentale;* a, "concomitant" cells. × 1,000

A comparison of the illustrations in Figures 4 and 5—sections of the peridia of *Cronartium ribicola* and *C. occidentale*, respectively—brings out the major differences between the two much more clearly than could any description. Particular attention is directed to the cells shown in Figure 3, B, *a*. These "concomitant" cells form a layer in connection with the peridium of *C. occidentale* which may correspond to one of the inner layers of the peridium in *C. ribicola;* but they resemble neither the peridial cells above them nor the spores below. The concomitant cells of *C. occidentale* are so highly refractive that they stand out very clearly when a fragment of the peridium is mounted with the under side toward the objective; and, as a matter of fact, they alone would probably serve to identify the species. If fragments of peridia of *C. ribicola* and *C. occidentale* are mounted side by side under the same cover the two appear to be absolutely distinct. Further work

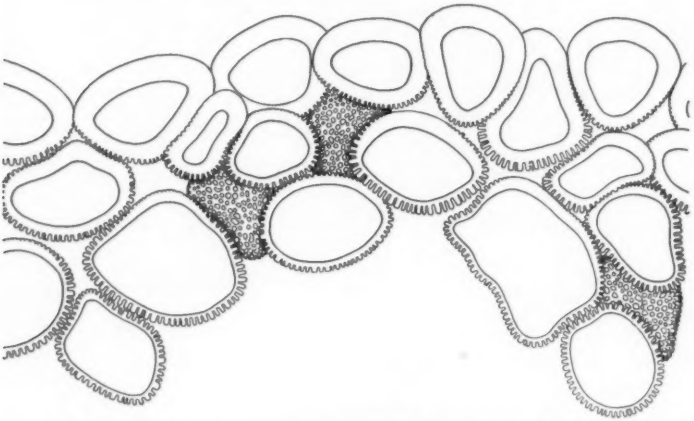

FIG. 4.—Outline drawing of a section of the peridium of *Cronartium ribicola* on *Pinus strobus*, cut near the central or dome region. × 585

will probably show peridial characters to be as important for the Peridermium group as Kern (*6*) has found them to be for Gymnosporangium.

BIOMETRIC COMPARISON OF THE PERIDIAL CELLS

Measuring the size of the peridial cells in surface view was found to be so difficult and the results so generally unsatisfactory that it was decided to use sections of the peridia for the biometric study, and to measure only the long and short dimensions of the outer layer of cells of the peridium, and the thickness of the outer wall of these same cells. In general the cells measured were in the roof rather than in the side walls of the peridia. The results are given in Table 3.

Biometric data on the size of the concomitant cells are given in Table 4. If these figures be compared with the figures for the size of the peridial cells and with the figures for the size of the aeciospores, it becomes evident that the concomitant cells are smaller than either. It is difficult to determine the size of the tubercles on the concomitant cells on account of their small size in end view, and because they are frequently compacted into irregular groups. They average something

less than 1μ in diameter in surface view. The obvious differences between these tubercles and the aeciospore tubercles are illustrated in Figures 1 and 3.

SIGNIFICANCE OF BIOMETRIC DIFFERENCES

The qualitative differences already described show rather clearly that the two rusts are different. To determine whether their morphologic differences are sufficient to warrant giving both of them specific rank, it is necessary to examine the significance of the differences which they exhibit in as many characters as possible.

The differences between the two rusts in the size of the outer layer of peridial cells are so large and consistent as to leave no doubt as to their reality. While the measurement data were obtained only from eight specimens for one species and five specimens for the other, two host species and at least two widely separated localities were represented for each fungus. The 100-cell length means from the different specimens all lie between 25 and 29μ for *Cronartium occidentale*

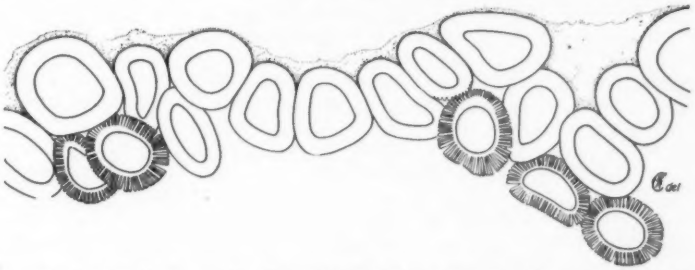

FIG. 5.—Outline drawing of a section of the peridium of *Cronartium occidentale*, cut near the central or dome region. × 585

and between 38 and 45μ for *C. ribicola*. The differences between the two species in the width and wall thickness of the peridial cells are of nearly the same order of decisiveness.

A glance at Table 2 shows decided differences in the dimensions of the surface view of the tubercles. For the seven specimens of *Cronartium ribicola*, from two hosts and two entirely different regions, the 100-measurement length means are all below 1.65μ; while for the five specimens of the other species, from two hosts and four localities, the length means are all above 2.2μ. The difference in tubercle width is less striking but apparently significant. It is evident that the populations which furnished these measurements of tubercles and peridial cells are quite distinct. Emphatic confirmation of the conclusion reached by inspection is furnished by the probability computations based on the measurements of these units. (See Table 5.)

In the case of the aeciospores themselves, the difference between the species is less obvious. The fact that the dimensions for different specimens overlap is best shown in Figure 2. The differences between the aeciospore species means are relatively much smaller than the differences which were found in the case of the tubercles and peridial cells. These points are evident after an examination of the probability constants shown in Table 5. The difference in

spore length between the two species means, though only 10 per cent, is more than 11 times its probable error, and on the face of things is undoubtedly significant. In spore width, wall thickness, and ratio $\frac{\text{mean length}}{\text{mean width}}$ the two species show less decisive differences with respect to their probable errors. In any case differences in these other characters do not furnish entirely independent support of the species difference, because all three factors are correlated with length. For *Cronartium ribicola* aeciospore lengths the correlation coefficient with width is 0.59 ± 0.09, and with wall thickness 0.61 ± 0.10. For *C. occidentale* lengths the correlation with width is 0.54 ± 0.11, with wall thickness 0.53 ± 0.13, and with the ratio of length to width 0.43 ± 0.12.

There remains the question as to whether the significant difference between the two populations sampled as to aeciospore lengths is really genetic, or simply due to differences in climate or substratum. These are questions on which the above probability figures give no information. The possibility that the larger spores of *Cronartium occidentale* are due to climatic influence can be practically ignored for the reason that it grows in a much drier climate than that in which the *C. ribicola* collections were made; and it is the general observation that the effect of drought, if any, is to decrease spore size. It is less easy to dispose of the possibility that the difference is due to substratum. So far as one can judge, if there had been more *C. ribicola* measurements from *Pinus monticola* the difference in average spore length between the two rust species would have been less. In the same way *C. occidentale* produced spores on *P. edulis* which differed less from *C. ribicola* than did those on *P. monophylla*. The aeciospore measurements are summarized separately by hosts in Table 6.

In the case of *Cronartium ribicola* it is impossible to reach definite conclusions as to the meaning of the difference between the spores from the two hosts, though all three of the specimens from *Pinus monticola* have larger spores than the mean of the spores from *P. strobus*, and the best probability computations possible under the circumstances indicate the difference to be significant. The specimens of *C. occidentale* were more evenly divided between its two hosts. The difference between the mean length of the aeciospores from *P. monophylla* and from *P. edulis* is scarcely more than twice its probable error. If the spores produced on *P. monophylla* are regularly larger, it is evident that measurements must be made from a larger number of specimens to establish the fact. Within each fungus species the difference between spore lengths from different hosts is small as compared with the difference in spore length between the species. The evidence is strong, though not absolutely conclusive, that irrespective of hosts, the spores of *C. occidentale* are longer than those of *C. ribicola*. To make it conclusive, it would be necessary to have measurements of each fungus from several congenial hosts. The ideal would be to have for comparison measurements of both fungi on a common congenial host, but it is very improbable that such a host exists. The wide spores and thick walls produced on *P. monticola* may indicate substratum effect, but perhaps they are the result of variation in sampling, of the moist atmosphere of Vancouver, or of an especially large-spored tendency in the strain of *C. ribicola* in

the single nursery-stock shipment which was the source of the Vancouver rust epidemic. Substratum effect is the most likely explanation, as the single specimen on *P. strobus* from Vancouver was decidedly below average in all the qualities in which the specimens from *P. monticola* were so high. There was striking agreement between the three *P. monticola* specimens in wall thickness; the possibility of poor sampling of the spores from the specimens measured was decreased by measuring 200 each from two of the specimens instead of the usual 100.

It will be noted that the probability constants in Tables 5 and 6 are computed from the population of specimen means, and not from the measurements of the individual spores. The latter procedure has been the one usually followed in the application of probability computations to spore measurements. It is theoretically at once evident that such a practice is unsound, for the reason that a sample composed of numerous spores from each of several specimens is not a simple sample such as one must choose for ideal probability computations. It is further evident that the number of individuals included in the sample is indicated not by the number of spores, but by the number of specimens. To substitute the number of spores for N in the denominator of the probable error formula will therefore give too low a value for the error. Empirical evidence of the lack of meaning of the probable error computed in the usual way from the individual spore measurements can be readily obtained from the data in the present paper. The error of the mean of the first 2,000 measurements of aeciospore lengths which were made for *C. ribicola* is 0.038μ. If this were valid for the species, the probable deviation of a mean of 100 measurements from the species mean should be $(2{,}000/100)^{\frac{1}{2}} \times 0.038\mu$ or 0.17μ. As a matter of fact, of the 20 specimen means, 17 show a larger deviation. Proceeding in the same way, it is found that 18 of the first 20 specimen means of *C. occidentale* differ from the species mean by more than the probable deviation (for this species 0.20μ). In fact, 11 of the 20 specimen means of the first fungus and 11 of the 20 means of the second actually differ from the species mean by more than three times the probable deviation figured from the 2,000-spore basis. In normal, simple populations only two such deviations would be expected in 47 submeans. It is undoubtedly true that means of 100 measurements taken at random from 20 or 25 different specimens would not so radically exceed the probable deviation; but it nevertheless appears that the only conservative procedure in calculating probability constants applicable to such a group as a species is to compute probable deviations or errors from a population of specimen means rather than from a population of individual measurements. In the present case the probable errors so computed are nearly four times as large as those obtained from the individual measurements, and the deviations of the specimen means quite closely follow the expectations based on these larger errors.

THE USE OF THE BIOMETRIC DATA IN THE IDENTIFICATION OF UNKNOWN SPECIMENS

Because the aecial stages of the two rusts have in the past inhabited different geographic regions and different hosts, there has been so far no need for microscopic identification of specimens. With the spread of *Cronartium ribicola* in the western part of the United States

the geographic criterion will no longer be valid, and it is entirely possible that either rust may be found capable of infecting one of the aecial hosts on which the other occurs. To avoid possible future confusion in the campaign which is being made for the control of *C. ribicola* in the West, it is desirable that there be available as many diagnostic criteria as possible. This is particularly true since some of the criteria may be changed as a result of change of host, and others, such as those dependent on the peridia, may be unavailable in weathered specimens. The size of the tubercles on the aeciospores is probably the character which may be depended on as most reliable and always available for examination. However, its determination requires very high magnification, and the aeciospores themselves will ordinarily be the character first measured. Because the differences between the two species in the dimensions of the aeciospores are small, the most probable diagnostic division point between them has been determined. Variability and probability constants based on the populations of specimen means—not of individual measurements—were employed in the determination. Obviously, if two species are equally variable the division point will be halfway between them; but if one species is more variable than the other, the division point should be farther from it and nearer the less variable species. The distance of the division point from each species mean should be proportional to the variability of the species. The division point located by the method described in the following paragraph is believed to have a higher probability of correctness than that determined by the inspection method used in earlier work with the uredinial stages of the two fungi (*3*).

The point is to be so located that the probability of a mean of measurements from a specimen of the smaller spored species being *above* the point would be equal to the probability of a specimen mean from the larger spored species being *below* the point. If A is the mean of the population of means for the larger spored species, B the mean for the smaller spored, σ_a the standard deviation of the A population, σ_b the standard deviation of the B population, and X the diagnostic division point, the conditions of probability referred to may be expressed by the formula

$$\frac{A-X}{\sigma_a}=\frac{X-B}{\sigma_b}$$

Solving for X[4]:

$$\sigma_b A-\sigma_b X=\sigma_a X-\sigma_a B$$

$$X(\sigma_a+\sigma_b)=\sigma_b A+\sigma_a B$$

$$X=\frac{\sigma_b A+\sigma_a B}{\sigma_a+\sigma_b}$$

Substituting the values for aeciospore length recorded in Table 1

$$X=\frac{(1.11\times 26.8)+(1.18\times 24.2)}{1.18+1.11}$$

$$=25.46$$

[4] The writers are indebted to T. R. C. Wilson for an improvement in the formula which they originally used for this purpose.

The probable deviation or error of a single specimen can be used in the formula in place of the standard deviation without affecting the result. The average deviation may also be used without serious loss of accuracy.

The diagnostic division point for aeciospore length, 25.46, is distant from the means 26.8 and 24.2 by approximately 1.66 times the probable deviations of single specimen 100-spore means, 0.81 and 0.76, respectively, for the two species. A deviation 1.66 times the probable deviation would be expected approximately 26 times in 100 trials, and only 13 times in the direction of the diagnostic division point. Assuming that the distribution approaches normal and that the specimens are in each case a good sample of their species, the chances are that about 13 per cent of all specimens diagnosed on the basis of the length mean would be diagnosed incorrectly.

The diagnostic division points obtained in this way for the other characters measured are shown in Table 5. For the ratios of length to width, variability and probability computations based on the logs of the specimen ratios would be somewhat preferable; but the variability in the ratios is small enough so that the differences in the results would be negligible. From the probability data, it appears that the chances of wrong diagnosis are approximately 36 per cent on the basis of width, 32 per cent on the basis of wall thickness, and 30 per cent on the basis of the $\frac{\text{mean length}}{\text{mean width}}$ ratio. The possibility of wrong diagnosis from the dimensions of aeciospore tubercles and of the peridial cells would be negligible.

Of course it must be understood that the bases for all these probability computations are inadequate in the number of specimens represented to justify entire confidence in them. The reliability of the figures given is also somewhat lessened by the fact that the specimens of each species have been treated as simple samples of a homogeneous population, when it is entirely possible that additional data would have shown that the measurements of each fungus are significantly affected by differences in host species. The diagnostic division points and the probability figures simply are the best results that can be condensed out of the available data. The actual number of specimens of the material investigated that would have been wrongly determined by application of the diagnostic division points is 6 out of 46 for aeciospore length, 16 out of 45 for width, 12 out of 33 for wall thickness, and 9 out of 45 for ratio $\frac{\text{mean length}}{\text{mean width}}$. None of the specimens whose aeciospore markings and peridial cells were measured would have been diagnosed wrongly on the basis of the dimensions of these structures.

The length, width, and $\frac{\text{mean length}}{\text{mean width}}$ data from Table 1 are shown graphically in Figure 2. Each plotted point represents both the mean length and the mean width of 100 measurements; in other words, the mean size of 100 spores. For example, the solid dot at the intersection of loci 24.4 and 17.9 represents the length and width means of *Cronartium ribicola* from Dunraven, N. Y. (See Table 1.) The stars represent the species means. The vertical line at 25.46 represents the diagnostic division for length, the horizontal line at 18.68 represents the point for width, and the solid diagonal line

drawn to satisfy the equation, length = 1.365 width represents the division for $\frac{\text{mean length}}{\text{mean width}}$. The figure serves much better than a table to illustrate the way the mean sizes of the spores from different specimens vary from the species mean and with respect to the diagnostic division lines, and the extent to which the ranges of the two species overlap. For biometric diagnosis, length appears from every viewpoint to be the most important of the aeciospore dimension criteria so far discussed, and if one were called upon to make a diagnosis of a white pine or nut pine Peridermium after an examination of the spore dimensions alone the length criterion would naturally be applied first.

A SINGLE DIAGNOSTIC INDEX WHICH USES BOTH LENGTH AND WIDTH

It will be noted that the line on Figure 2 which represents the most probable separation of the two species on the basis of spore length is vertical, that for width is horizontal, and that for the ratio of length to width has its origin at 0 for both length and width. It is at once evident that the line which will best separate the two "swarms" of specimen means shown in the graph will rarely happen to be an exactly horizontal or vertical line, or one which starts at zero for both length and width. It is evident from the graph, and has been confirmed by the coefficients given on page 524, that length, width, and their ratio are so correlated within each swarm that the criteria are not independent, and there is little advantage in trying to employ them separately for diagnostic purposes. In such cases the writers recommend that the line which seems best to separate the two swarms be located by inspection. The broken diagonal line in Figure 2 was so located. For all points on the line, length is $15.5\mu + 0.553$ width. This value was found after the line had been located, by noting that it cut the zero width line at 15.5 on the length scale, and increased 0.553μ on the length scale for each 1μ increase on the width scale. Its significance is that if a specimen has spores whose average length is more than $15.5\mu + 0.553$ of their average width, it is considered as probably *Cronartium occidentale;* if the average length is less than this value, the specimen is probably *C. ribicola.* Of the 45 specimens for which both lengths and widths are available, it will be seen on the graph that 4 would be wrongly identified by this criterion. For this particular lot of data, the vertical diagnostic length line happens to separate the swarms nearly as well as any other line which can be drawn.

While the slope of this line has been decided by inspection, it has been pivoted on the intersection of the most probable division values for length and width, and is probably more accurate than if located entirely by inspection. Its slope could be determined mathematically by describing for each swarm a contour of the best fitting frequency surface for that swarm. The points at which the contours for the two swarms intersect would be loci of the most probable, or at least of a very probable, straight line of separation. It is difficult to imagine any situation in mycological work which would justify the amount of computation required by such a process.

Where the correlation between length and width is not rectilinear, or where the two swarms differ materially in shape or degree of spread

the best line of separation of two swarms will be a curve. The determination of the best curved line would be a still more difficult matter, and the advantage over a straight line, if any, would probably be slight.

GENERAL DISCUSSION

One of the populations which have been compared in the present paper has included Cronartium individuals occurring on a 5-needled pine in the northeastern part of the United States, together with a smaller population on this and another 5-needled pine in British Columbia. The other population has included Cronartium individuals which grow on two nut pines in the southwestern part of the United States. The two populations were at one time confused; they were classed as separate species, chiefly on the ground of differences in host preference. In an earlier paper one of the present writers has shown that there is a small quantitative morphologic difference between the two fungi in the urediniospores when grown under outdoor conditions, whether in their respective natural habitats, or as a result of inoculation on the same hosts and under the same conditions. The data in the present paper show that the aecial stages differ very strikingly in some morphologic characters, and in others display smaller differences which by biometric methods can be shown to be significant. The differences which are so large that biometric demonstration is not required are of course the most important ones from every standpoint. It has not been possible in the aecial-stage comparisons to eliminate the possible influence of different climate or host, but the evidence indicates that the major portion of the differences observed are genetic, rather than the effect of environment or substratum. Added to the differences which had previously been demonstrated (*5*), these aecial differences seem sufficient to end any question as to the independent specific rank of *C. occidentale*.

These morphologic differences in the aecial stage should also be helpful in situations which may require the determination of specimens from unidentified host bark, or where there is suspicion that one of the species has jumped to a host outside of its usual host range. It is well to point out, however, that unless methods strictly comparable to those used in this paper are employed, other investigators can not expect the means and diagnostic division points to hold good, and they will have to rely on the qualitative morphologic differences for diagnosis.

The following descriptions of the aeciospores and peridia of the two forms combine the morphologic and biometric data. The figures are the same as, or are based on, the averages of the populations of means shown in Table 1:

Cronartium ribicola Fischer—

Peridia thick, persistent; 3 to 5 cells thick; outer layer of cells in region near dome, sectional view, long and short dimensions (basis 100 measurements from each of 8 specimens) 41.4 by 28.5μ, standard range [5] of the 8 specimen means, 39–43.8 by 26.3–30.7μ; outer wall of cells in outer layer smooth 7.16μ thick, generally thicker than inner wall; inner wall marked with short

[5] The lower and upper limits of the standard range differ from the mean by the amount of the standard deviation. Ordinarily about two-thirds of the specimens should have measurements within the standard range.

tubercles; outer walls of cells in second and third layers sometimes smooth, sometimes minutely warted, inner walls marked with tubercles; walls of cells in the inner layers generally uniformly marked with tubercles; walls progressively thinner from the outer layer to the inner layer of the peridium; outer walls of cells in first two or three layers usually thicker than the inner walls.

Aeciospores obovoid to ellipsoid, generally smoothly curved in outline, (basis 100 spores from each of 25 specimens) 24.2 by 18.3μ, standard range of the means 23.1–25.3 by 17.2–19.4μ; ratio mean length divided by mean width, geometric mean of the values for the 25 specimens 1.32; wall (basis 100 spores from each of 19 specimens) 3.41μ; wall partly smooth and partly marked with tubercles, the smooth area fissured near junction with tubercles; tubercles fairly regular in outline in end view (basis 100 measurements from each of 7 specimens), 1.52 by 1.10μ, standard range of the means 1.45–1.59 by 1.06–1.14μ.

Cronartium occidentale Hedg. Bethel, and Hunt—

Peridia thin, evanescent; one cell, rarely two cells thick; outer layer of cells in region near dome, sectional view, long and short dimensions (basis 100 measurements from each of 5 specimens) 27.4 by 19.1μ, standard range of the means 26.3–28.5 by 18.2–20.0μ; outer wall of outer layer of peridial cells 4.68μ; outer wall of cells in outer layer smooth, inner wall generally smooth, occasionally marked with minute tubercles; "concomitant" cells just below outer layer of cells of peridium (basis 100 measurements from each of 5 specimens) 21.9 by 17.9μ; standard range of the means 21.6–22.19 by 17.5–18.3μ; wall of concomitant cells 4.60μ; tubercles minute in end view, averaging somewhat less than 1 by 1μ, often compacted into irregular clumps.

Aeciospores obovoid to ellipsoid, often somewhat irregular in outline (basis 100 spores from each of 20 specimens) 26.8[6] by 19μ; standard range of the means 25.6–28 by 18–20μ; ratio mean length divided by mean width, geometric means of the values for the 20 specimens, 1.41; wall (basis 100 spores from each of 14 specimens) 3.85μ; wall partly smooth and partly marked with tubercles, the smooth area fissured near junction with the tubercles; tubercles irregular in outline in end view (100 measurements from each of 5 specimens) 2.29 by 1.33μ; standard range of the means 2.23–2.35 by 1.31–1.35μ.

A comparison between Figure 2 of the present paper and Figure 4 of the urediniospore paper (*3*) reveals the fact that the urediniospores and the aeciospores of *Cronartium occidentale*, respectively, differ in size and shape from the urediniospores and aeciospores of *C. ribicola* in the same general way. Reduced to percentages based on mean spore size, the urediniospores of *C. ribicola* may be described as 93 per cent as long as those of *C. occidentale*, and the aeciospores of *C. ribicola* as 90 per cent as long as those of *C. occidentale*. Whether this same general relation holds for the teliospores and sporidia remains to be seen.

None of the measurement distributions encountered gave indications of real bimodality. Skewness was more commonly positive; no extreme tendency to skewness was seen.

In the case of two forms which are as close together in their host relationships and morphology as *Cronartium ribicola* and *C. occidentale*, extreme spore-size ranges are utterly useless for diagnostic purposes. The statement in an earlier paper (*1, p. 632*) that the mature aeciospores of *C. ribicola* measure "18 to 21 by 20 to 26μ" tells only part of the story. In the present study, for instance, the extreme ranges for *C. ribicola* and *C. occidentale* are 16–43 by 11–25μ and 19–40 by 9–28μ, respectively. In which species would spores described by the first range cited be placed? The critical case presented by these two Cronartium species serves excellently to illustrate the fact that averages are more useful than ranges in certain types of taxonomic work.

[6] Length based on 21 specimen means.

SUMMARY

General observations have indicated marked differences in the habit of the aecia of *Cronartium ribicola* and *C. occidentale*. In the former the aecia are usually distinct sori with persistent peridia; but in the latter the aecia vary from distinct sori, e. g., on *Pinus monophylla*, to broad spore-bearing layers under the bark, e. g., as in infections on *Pinus edulis*, with thin inconspicuous peridia.

The aeciospores of *Cronartium occidentale* are slightly longer and wider than those of *C. ribicola;* they have a slightly thicker wall, a more irregular outline, and larger and more irregularly distributed tubercles.

The peridia of *Cronartium ribicola* are 3 to 5 cells thick, all the cells being marked with tubercles; the peridia of *C. occidentale*, on the other hand, are rarely more than 1 cell thick, and the cells in this one layer are rarely marked with tubercles. Certain cells, unlike the peridial cells or the aeciospores, are located just beneath the peridium of *C. occidentale*. These cells are given the name "concomitant cells."

Any unweathered specimen of *Cronartium occidentale* can be distinguished from any specimen of *C. ribicola* by any one of the following characters: Length, width, or shape of aeciospore tubercles; length or width of outer peridial cells; smoothness of outer peridial cells; and the contrast between concomitant cells and the cells above and below them. In aeciospore lengths, and in the gross characters of the sori, it is usually possible to distinguish between the two species, but in occasional specimens the two rusts overlap in these two characters.

The morphologic differences between *Cronartium ribicola* and *C. occidentale* seem quite sufficient to justify their standing as separate species.

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BURRKNOT FORMATIONS IN RELATION TO THE VASCULAR SYSTEM OF THE APPLE STEM [1]

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INTRODUCTION

Knight (*8*)[3] in 1809 mentioned the occurrence on the stems of certain varieties of apples of "rough excrescences, formed by congeries of points which would have become roots under favourable circumstances." Recently the writer (*11, 12, 13*) has called attention to the work of Knight and of others and has shown that many of our present commercial varieties of apples can be grown from cuttings containing burrknots, as Knight called these rough excrescences. The present paper deals with the anatomy of burrknot formation, a study undertaken in order to throw more light upon the physiological problem of the formation of adventitious roots on stems.

Kissa (*7*) in 1900 briefly described a case of "goiter gnarl" on *Malus chinensis* (*Malus spectabilis* Borkh.?), which was undoubtedly burrknot and which involved the production of adventitious roots. He showed that such roots arise as swellings at the outer edges of medullary rays, that growth in length of the tips is stopped by dryness, and that a lateral, basal branching takes place within the protecting cortex of the stem. Kissa made no reference to any definite location of these roots in relation to the primary vascular structure of the stem.

Borthwick (*2*) in 1905 published descriptions and photographs of adventitious roots in the apple and the maple, as well as in *Pyrus aucuparia*, *Ulmus campestris*, *Thuya gigantea*, and *Cupressus pisifera* (*Chamaecyparis pisifera*?). He shows that such roots arise at the outer edges of medullary rays, and that the cambium of the stem is connected with the cambium of the root, but he makes no mention of the relation of these roots to the primary vascular system.

Van der Lek (*9*) seems to have been the first to recognize the relation between the primary vascular system and the distribution of "root germs" on woody plants. Working with willow, poplar, and black currant, he found that most of the numerous root germs—he found 36 in one internode—arise in definite relation to the primary vascular system. He distinguishes between nodal roots—those which actually arise on the leaf traces, or branch traces themselves—and internodal roots—those which arise on the medullary rays. In some species the root germs are largely internodal, in others chiefly nodal.

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[2] The writer desires to express his appreciation to Prof. Duncan S. Johnson, of Johns Hopkins University, for the many helpful suggestions offered throughout the course of the work here reported and for supplying the necessary equipment and materials.

[3] Reference is made by number (italic) to "Literature cited," p. 544.

METHODS AND MATERIALS

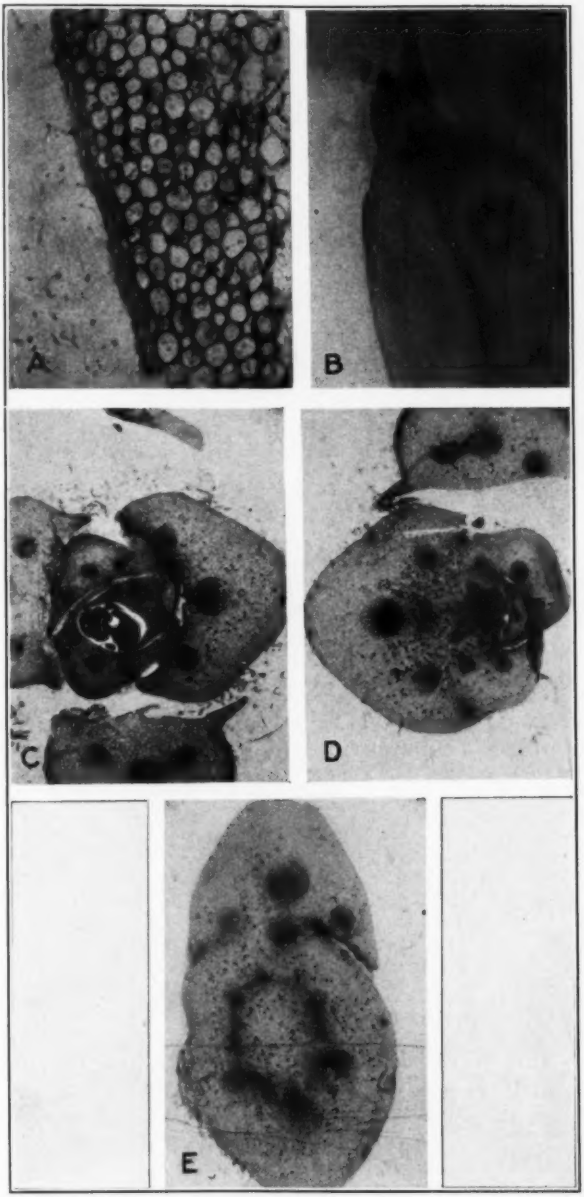
The work here reported was done in the botanical laboratory of Johns Hopkins University, Baltimore, Md. For the study of root anatomy, material of the apple varieties Buckskin, Jersey Sweet, Rambo, Wagener, and Springdale was used. The material was removed from 25-year-old trees in the orchard at Arlington Experiment Farm, Rosslyn, Va., cut into ¾-inch lengths, and placed in Bouin's picric acid-formalin-alcohol killing fluid. The air was removed under vacuum to insure the penetration of the killing fluid. After 24 hours the material was washed in 40 per cent alcohol, which was changed several times during two or three days. The material was then kept in 60 per cent commercial hydrofluoric acid for two to four days, or until it was soft enough to cut easily with a knife. It was next washed in water until no hydrofluoric acid taste could be detected. The material was next run up through the alcohol. Since it was desired to show cell walls rather than cell contents, a more rapid transition was used than is desirable for cytological work. It was necessary to thoroughly remove all water by changing the absolute alcohol several times. The total time in the various alcohols was usually about five days.

The hydrofluoric-acid treatment necessary to soften 5 to 10 year-old wood made the material so brittle that it was necessary to embed it in celloidin. Plowman's method, as given by Chamberlain (*4*), was used. However, in order to determine the exact position of the root germs in relation to the leaf and branch traces, it was absolutely necessary to have serial sections. To get these a modification of Wilhelmi's (*14, p. 17*) method was used. After the celloidin-embedded material had been hardened in chloroform it was put into xylol (which was changed several times) and then embedded in paraffin. This made it possible to cut serial sections on a rotary microtome and to get sections of 8 to 10 microns in thickness of apple stems 10 years old. By putting the ribbons containing the sections on the slide and keeping them in place with mosquito netting, tied on with thread, it was possible to stain in series on the slide. Safranin and gentian violet proved the best stains. Some study was made of free-hand sections of fresh material. But for securing complete thin sections of wood the double embedded method was found to be necessary.

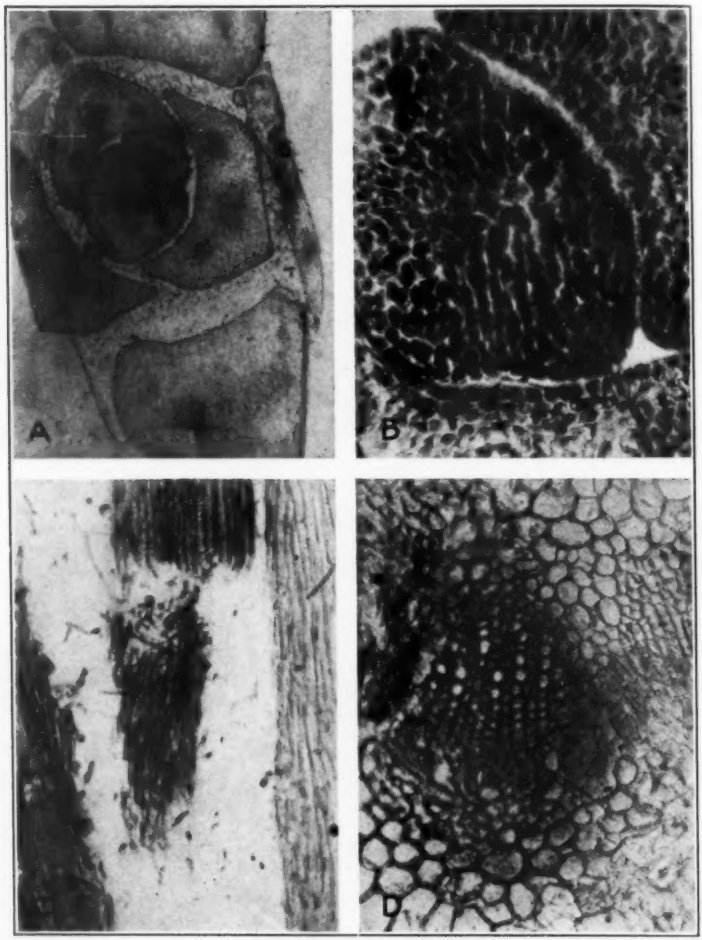
The structure of the primary vascular system was studied in new growth taken from the varieties listed above and also from the varieties Delicious and Tolman. Paraffin sections and free-hand sections were used. In the latter case the bundles were traced by haematoxylin which had been allowed to enter the twigs used, either at the cut end of one of the petioles or through the cut base of the twig. In both instances the evaporation from the leaves furnished a pull which drew the stain into the stem. The haematoxylin was found to follow closely the leaf-trace bundles, showing very little lateral diffusion.

THE PRIMARY VASCULAR SYSTEM OF THE APPLE

Except for the short hypocotyl, the entire stem of the apple tree is the product, primarily or secondarily, of the terminal growing point of the embryo. This apical growing point of the stem is quite



A.—Origin of cork cambium in epidermis. (× about 150)
B.—Longitudinal section through stem growing point. (× 15)
C.—Cross section of stem of Buckskin apple, just above the growing point. (× 15)
D.—Another cross section through the stem growing point. (× 15)
E.—Just below the growing point of the stem shown in C and D. (× 15)



A.—Cross section of the growing point of stem of Wagener apple. Note the procambial strands in the leaves near the center and the vascular bundles, which arose as procambial strands, in the outer leaves. Notice also the protecting hairs. (× about 20)
B.—A higher magnification of part of the stem growing point shown in A. (× 150)
C.—Spiral thickening of primary xylem elements. (× 100)
D.—Origin of cambium within the bundle. Note the metaxylem in radial rows. (× 100)

effectively protected from drying out by the leaves recently formed by the growing point. These leaves grow very rapidly and soon push up and entirely surround the growing point. As will be noticed in Plate 1, B, C, and D, and Plate 2, A, the young leaves bear many hairs.

From this terminal growing point are differentiated the promeristems, from which the tissues of the main stem itself are derived. It also forms the leaves and the lateral branch rudiments or axillary buds. Before primary growth has entirely ceased there has been formed a cambium ring which is to give rise to all secondary vascular tissues. At about this same time the cork cambium, from which the outer portion of the bark is derived, is formed in the epidermis (pl. 1, A). The first cork cambium in most woody dicotyledons is derived from the outer layer of the primary cortex, although not a few genera have a cork cambium initiated in the epidermis, as does the apple.

The terminal growing point of the apple is broad and depressed (pl. 1, B), so that within a few millimeters of the tip the hollow central cylinder or siphonostele, characteristic of most woody dicotyledons, is recognizable. A cross section taken immediately behind the growing point (pl. 1, E) shows pith in the center with the vascular bundles grouped in a ring between a central pith and the cortex. Connecting the central pith and the cortex, and separating the bundles from each other, are the medullary rays. The cells of the central pith, the medullary rays, and the cortex differ from each other chiefly in position. Practically all cells of the stem for several centimeters back of the growing point, even cells of the central pith, contain chlorophyll.

Each bundle of the stem consists of a longitudinal strand of cells. The bundles have no direct connection with the growing point, but as they run upward each passes out of the stele and then on through to the outer cortex, where, coming close to two other similar bundles, it enters the base of the petiole. Here the two outer bundles divide, the inner half of each continuing out into the leaf, the outer half going into a stipule. Followed downward, each stem bundle becomes smaller and smaller in the tangential dimension until—several internodes below the point of its entrance—it merges with a neighboring bundle derived from another leaf. The following description of the leaf traces of woody dicotyledons in general, given by Eames and MacDaniels (*6*), is applicable to the special case of the apple.

> Since the trace is merely an extension of the vascular system of the stem—either as a definite, abruptly separated branch of that system, or as a strand gradually set off as a distinct part—there is not usually a definite point of origin of a trace. The trace, as an identifiable, protoxylem-containing strand may, indeed, often be followed down the stem for some distance below the level at which it begins to swing outward, and there found to merge with other traces or with the xylem of the primary cylinder. . . . Below the point where the trace is separated from the cylinder it is in many cases evident for some distance as a distinct, though not isolated, strand, chiefly of protoxylem. This distinctness of the strand in the xylem cylinder is due to the type and size of cells composing it, these being different from those of the adjacent xylem. . . . Such a downward extension of the trace may be very short or lacking, or may be several internodes in length.

Figure 1 shows diagrammatically the path of the bundles from 9 leaves in a stem of Delicious apple. It will be noted from the diagrams that the general arrangement is such that at any given cross section of the stem the 9 most distinct bundles are the 3 sets

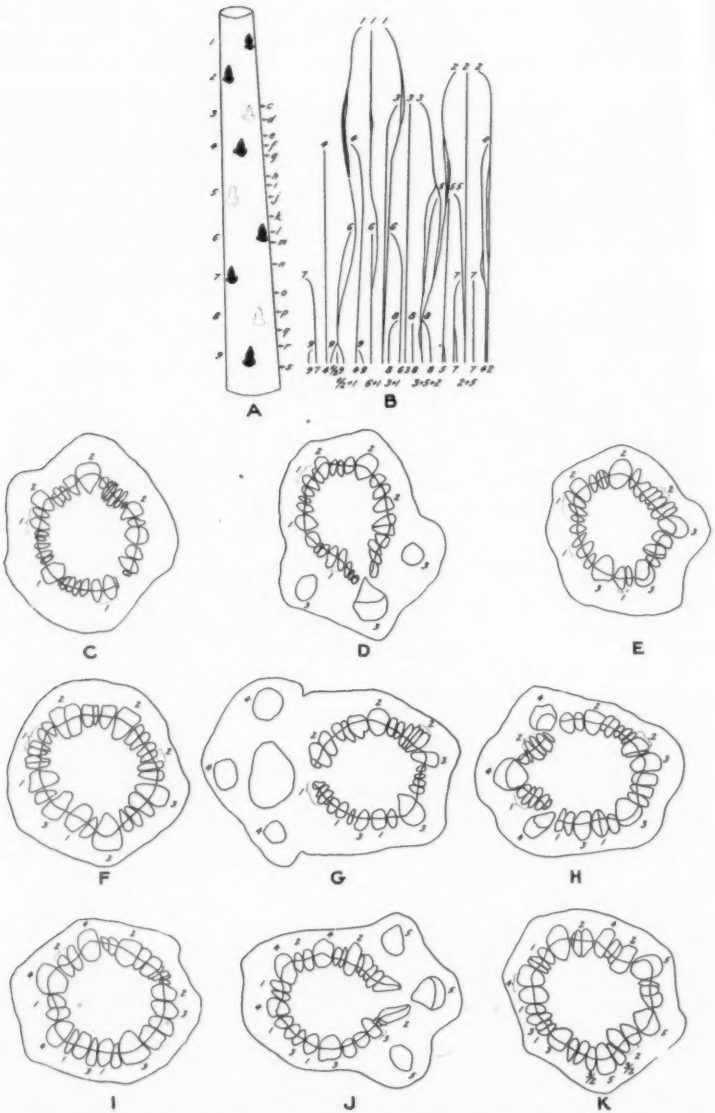


FIG. 1.—Diagrammatic representation of the courses taken by leaf traces in a stem of Delicious apple. A, external appearance of the stem. The numbers refer to the numbers of the buds, the letters to the points at which cross sections having the corresponding letters were cut. B, diagrammatic representation of the paths of the bundles. C to S, camera-lucida sketches of cross sections taken at points indicated by the corresponding letters in A

of 3 each which lead to the 3 leaves next above the section. These bundles are so distributed that one of the side bundles of the uppermost of the 3 leaves (numbered 1 in fig. 1, D) lies between a lateral bundle and the median bundle of the leaf immediately above the section (numbered 3 in fig. 1, D). From the successively lower sections (E to S) it will be seen that the bundles become less and less prominent and that each will ultimately lose its individuality by fusing with a neighboring bundle. This can be clearly seen by following two of the bundles of leaf 2 in diagram B, and in sections C to S. (The third bundle of leaf 2 fused still lower down.)

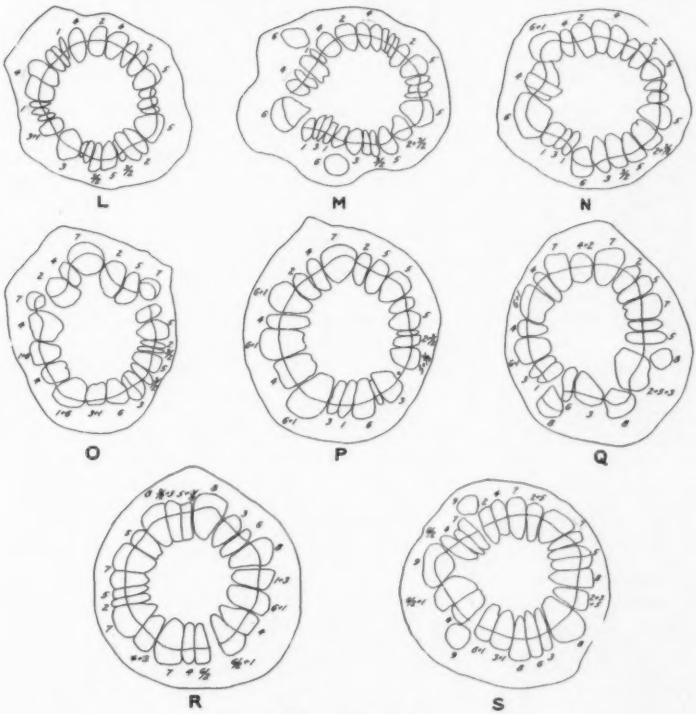


FIG. 1—Continued

Figure 2 shows a series of sections through a Delicious apple stem in which interposition of bundles does not always occur. In rare cases the three traces from each of the three leaves immediately above the section lie next each other in the stele. The laterals of one set do not cross over to become interposed between the laterals and medians of the adjoining set. (See trace 3 at right in diagram B, fig. 2, and sections C to K.)

In other cases, the upper bundle divides, half going on each side of the lateral bundle from the second leaf below. (See trace 2 in fig. 2, B.) In both these sets of diagrams (figs. 1 and 2) it will be

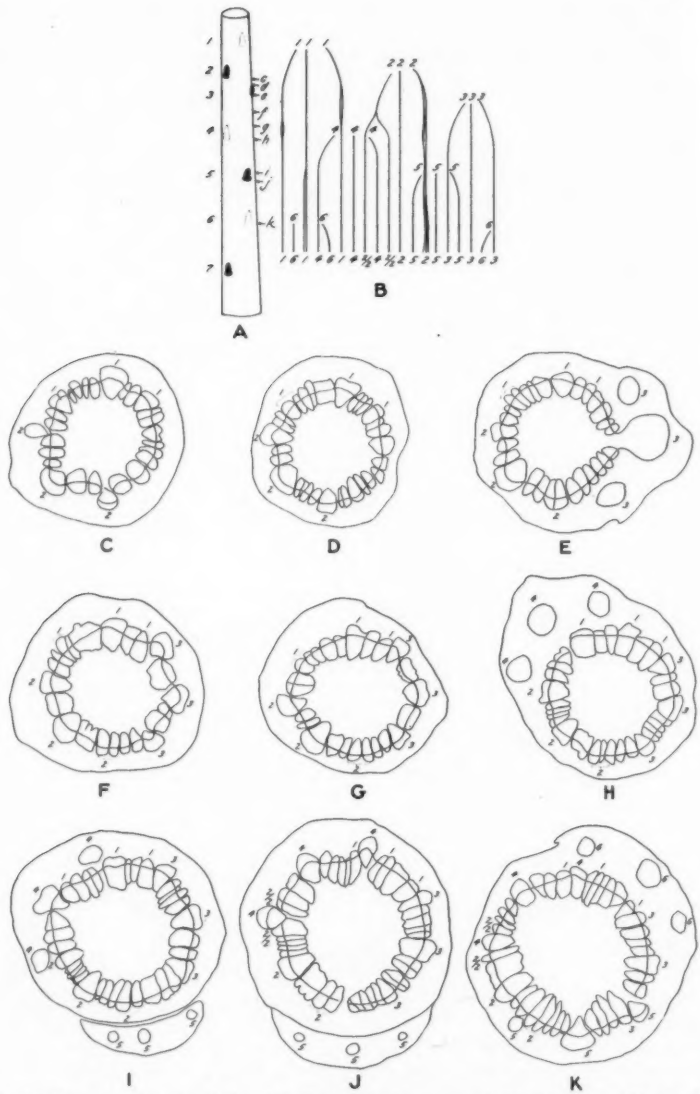


FIG. 2.—Diagrammatic representation of the three types of arrangement of leaf traces found in the stem of a Delicious apple. A, external appearance of the stem. The numbers refer to the numbers of the buds, the letters to the points at which cross sections having the corresponding letters were cut. B, leaf-trace courses. Note that between 3 and 1 there is no interposition such as occurs between 3 and 5. Note also that one of the laterals of leaf 2 divides and goes on both sides of the lateral of leaf 4. C to K, camera-lucida sketches of cross sections taken at points indicated by the corresponding letters in A

noticed that there seems to be little regularity in the way the bundles divide and merge. These irregularities seem surprising in view of the fact that only a primary vascular structure is under consideration and that supposedly it is the genetic constitution, rather than any controllable outside factor, that determines the primary arrangement peculiar to any given species.

As is mentioned below, in connection with the spiral arrangement of leaves on the stem, these irregularities in the courses of the bundles may possibly be significant in regard to the root formation or fruit-bud differentiation or to other factors, though of this nothing is known at present. Nägeli (*10*) noted and diagrammed similar irregularities of the bundle courses in Ribes, Prunus, and in other plants, without commenting on their significance.

The fact that each bundle takes an almost direct longitudinal course and the further fact that merging takes place only after the bundle has become much reduced in tangential width would seem to offer at least a partial anatomical explanation for the physiological results obtained by Auchter (*1*), which led him to conclude that there was normally little or no transverse movement of materials in the apple stem.

After the first differentiation of the leaf rudiment beside the growing point, the bundles of the leaf are laid down as procambial strands. Each procambial strand consists of a group of meristematic cells, each of which divides so that most of the cells are much smaller than those of the undifferentiated part of the growing point. Plate 2, A and B, shows procambial strands formed near the growing point.

By continued division and growth of cells the procambium forms the first water-conducting cells, the protoxylem. These are next to the pith and are closely followed in position and time of development by the metaxylem. Both the protoxylem and the metaxylem persist throughout the life of the plant or until decay sets in at the center of the trunk. These primary xylem elements probably function for many years, although it is only a few weeks before they are in large measure replaced by larger and more efficient tracheids and vessels formed by the cambium. In Plate 2, C, is shown the spiral thickening of the primary xylem with extremely thin walls between the thickened spirals where the thickening bands of some of the elements have been pulled out in sectioning, and resemble very small springs. The larger elements of the metaxylem are pitted and are indistinguishable from secondary tracheids. The apple, unlike most woody dicotyledons, has the primary xylem elements lined up in definite rows, with rows of parenchyma between them. This arrangement makes it difficult, in the stem, to distinguish the metaxylem from the secondary xylem. In the leaf, where no cambium is found, one sees that these same radial rows of metaxylem occur entirely independently of the secondary xylem although they look very much like it.

At about the same time that the protoxylem and metaxylem are being differentiated there arise on the outside of the bundle the protophloem and metaphloem. These differ in appearance from the other cells of the procambium chiefly in being of much smaller diameter. They are living cells, with the function, supposedly, of conducting elaborated food, although this function of the phloem has never been definitely proved, and Dixon (*5*) and others have raised

serious doubts concerning the conduction of elaborated food by the phloem. The primary phloem cells are soon crushed by the outward growth of secondary phloem, cut off by the cambium which arises between the metaxylem and the metaphloem.

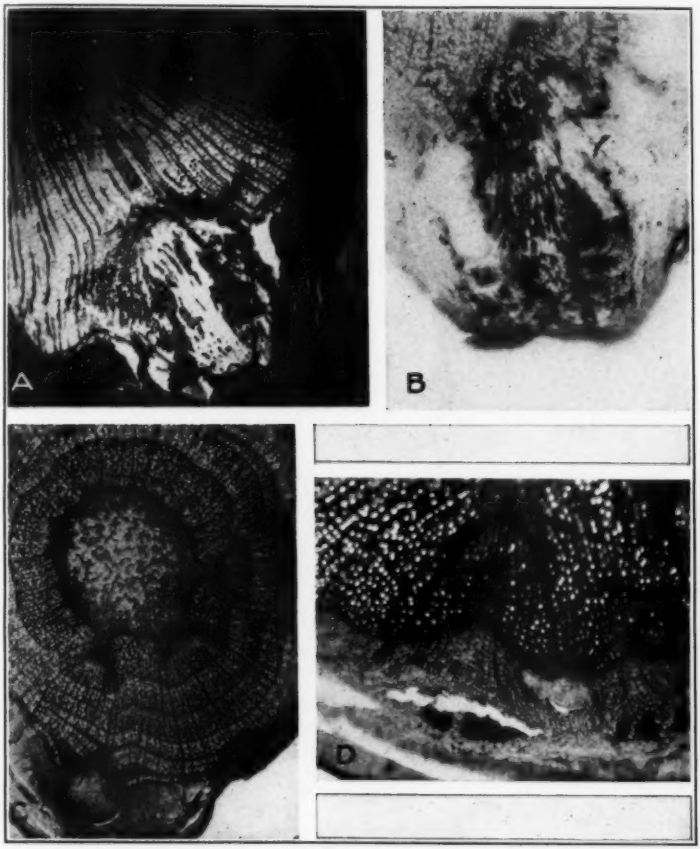
In the axil of each leaf either a fruit bud or a branch bud is formed by the growing point. This bud is connected with the pith by a broad band of parenchymatous cells. In contrast to the leaf gaps, the branch gaps extend downward only a short distance. The lateral bud is in all essential respects a duplicate of the terminal bud, but it may or may not have the same leaf spiral that the terminal bud has. If one examines a branched shoot of the apple he will see that all the buds and leaves formed by any one growing point stand in definite relation to one another. Starting at any given leaf and going up the stem through the leaf bases in the order of their origin, one makes two revolutions around the stem, and passes four leaves, before arriving at the sixth leaf, which is directly over the first leaf. Thus the apple is said to have a 2/5 phyllotaxis. It will be noticed, however, that in part of the stems the twist—i. e., the direction of the genetic spiral—is clockwise, while in other stems this spiral joining the leaves in the order of their development runs counterclockwise. It seems improbable that any significant correlation exists between root germ or fruit-bud formation and the direction of the genetic spiral or the twist of the stem. However, it is possible that such a correlation exists, and this point should be investigated.

Between the metaxylem and the metaphloem is a region, at first undifferentiated, but which soon gives rise to the cambium in that portion of the bundle attached to the stele (pl. 2, D). A cambium exists as such for only a very short time, if at all, in that part of the bundle that passes out to the leaf. From the cambium, xylem cells are cut off on the inside and phloem on the outside. Between the vascular bundle strands there is formed the interfascicular cambium, which cuts off parenchymatous medullary ray cells on both sides. Thus, a cross section such as Plate 1, E, shows a complete cambium ring. The cambium layer as a whole forms a hollow, truncate cone, with short breaks at the leaf and branch gaps.

BURRKNOT FORMATION

The majority of burrknots swelling on apple twigs are shown by superficial examination to occur in the immediate vicinity of a bud. Probably this fact led Knight (*8*) to conclude that such burrknots had the power of giving rise to *either* roots or shoots. Since both root and stem growing points are present at the nodes, of course both may arise from almost the same place, although each rudiment has a distinct growing point that probably can develop into only one or the other.

As is shown by Figure 1, C to S, and Figure 2, C to K, the bands (or radial plates) of parenchyma connecting the cortex with the central pith, are, beginning with the broadest: (1) The branch gaps; (2) the leaf-trace gaps; (3) the pair of primary rays, one on each side of each leaf trace after it has entered the stele; (4) the secondary rays within the individual leaf trace bundles. Examination of thousands of sections has led to the conclusion that the order in which they are listed above is also the order of frequency of oc-



A.—Root germ at node. Note the broad band of parenchyma, characteristic of the branch gap, connecting the root germ with the central pith. (× about 25)
B.—Root germ, 5 years old, which has arisen on a leaf trace. (See also fig. 3.) (× 25)
C.—Photomicrograph of cross section shown in diagram in Figure 4, A. (× 10)
D.—Very young root germ on primary ray of stem of Buckskin apple. (× 25)

currence of root primordia on the four types of rays of apple stems. Figure 3 shows diagrammatically the four ways in which root germs may be placed. Not a single case has been observed on the apple in which roots arose from xylem cells or even phloem cells. Thus the root germs are always placed with definite relation to the primary vascular system, except when they occur on secondary rays.

In the initiation of a root germ, one of the cells in the vertical sheet of parenchyma making up the medullary ray, branch gap, or leaf gap increases its meristematic activity and cuts off a great many cells, such increased activity starting at the point in the ray where it crosses the cambium. No physiological explanation of this strict localization of the root rudiment can yet be suggested. The root germ is soon recognizable in sections as a knob of meristematic tissue. By the end of the second or third year these knobs have elongated and are then roots, with root cap, growing point, cambium, and primary xylem all discernible, as was pointed out by Kissa (7). Apparently the branching of the root is very slight until after the third year. Branching may continue indefinitely, so that on old trees most of the surface of large limbs may be covered with these burrknots. The age at which burrknots appear differs in different varieties. The size of individual root germs, the size of patches of burrknots, and the amount of callus around each root germ also differ.

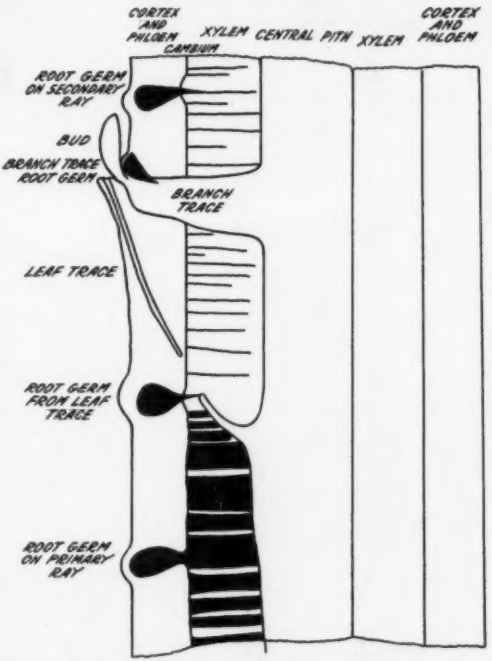


FIG. 3.—Diagrammatic representation of a radial section of apple stem showing the four ways by which root germs may arise

Plate 3, A, shows a root germ which has arisen on the branch trace. Plate 3, B, shows a leaf-trace root germ several years after differentiation. The root germ has appropriated the vascular system of the leaf trace. This is shown in the diagram given in Figure 3. Figure 4 and Plate 4 show various stages in the differentiation of root germs in the medullary rays bounding the leaf traces and in secondary rays.

WOOLLY APHIS GALLS

Brown (*3*) has called attention to the fact that burrknot swellings might possibly be caused by woolly aphis (*Erisoma lanigera* Hausmann). The writer (*11*) has already published some evidence against this view. His further anatomical work strengthens these objections. A study was made of sections of many twigs which showed superficially the characteristic woolly aphis swelling—a swel-

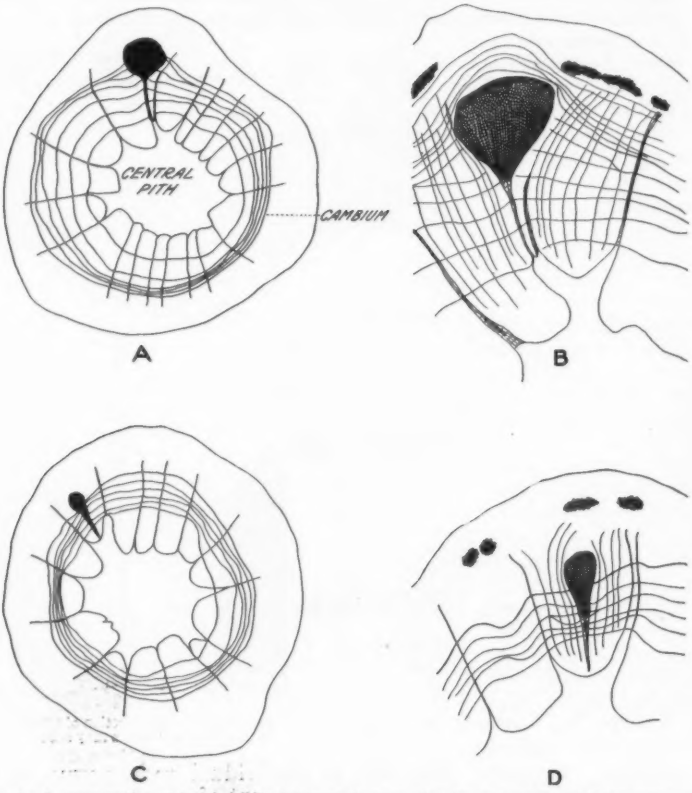
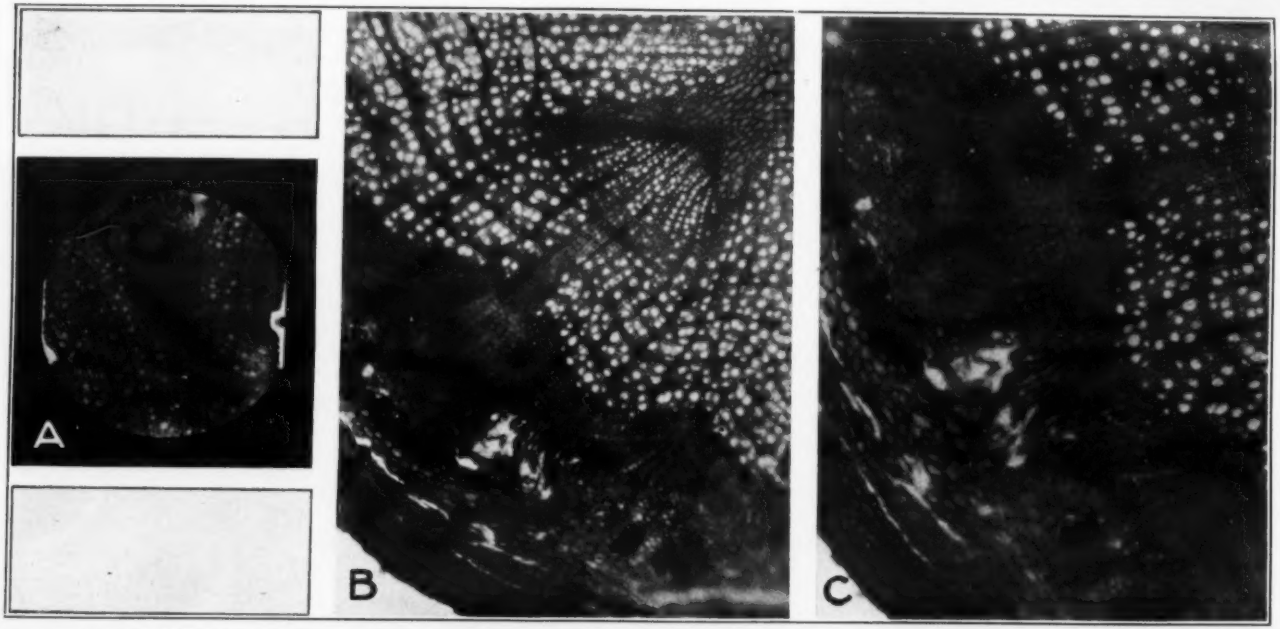
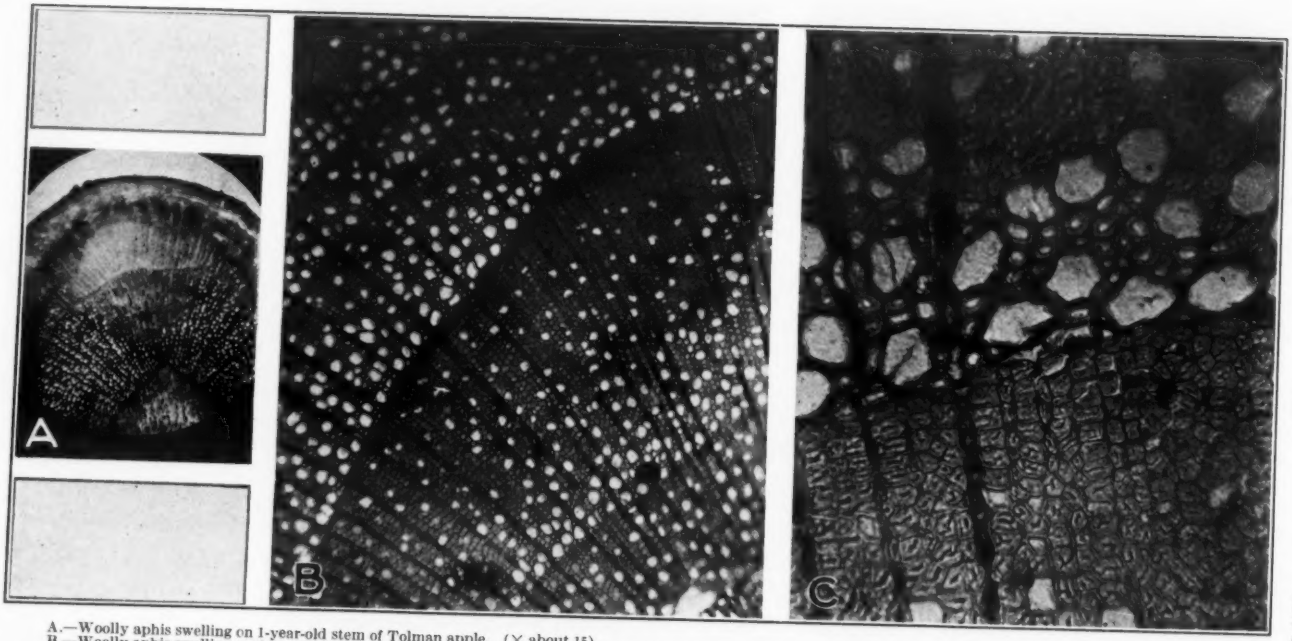


FIG. 4.—A, cross section of 6-year-old Buckskin apple stem, showing root germ which has arisen on a primary medullary ray; B, the same root germ as that shown in A and in Plate 3, C; C, cross section of 6-year-old stem of Buckskin apple, showing root germ which has arisen on a secondary medullary ray; D, the same root germ as that shown in C

ling of a type quite distinct from burrknot swellings. Such (assumed) woolly aphis swellings were taken from the stems of Buckskin, Tolman, and other varieties of apples. Among the varieties at Arlington Farm, the Buckskin showed the most pronounced burrknots, while Tolman has never shown them. The (assumed) woolly aphis galls were about equally numerous on both varieties. As is shown in Plate 5, anatomically the woolly aphis swellings have no points of close resemblance to burrknots. The peculiar anatomical charac-



A.—First cell division at base of old root germ. Note that the root arises from one cell of the right row of the primary medullary ray, the left row of cells being pushed aside by the more rapid growth of the root germ. (× about 50)
B.—Root germ arising on a secondary ray. (See fig. 4, C and D.) (× 30)
C.—Same cross section as that shown in B and in Figure 4, C and D. (× 50)



A.—Woolly aphis swelling on 1-year-old stem of Tolman apple. (× about 15)
B.—Woolly aphis swelling on 6-year-old stem of Buckskin apple. Note the very great local swelling of the stem caused by the great increase in the number of fiber tracheids at the close of the second year's growth. Note also the medullary rays that apparently were not changed (except in length) by the woolly aphis. (× 50)
C.—A higher magnification of part of B. (× 200)

teristic of the woolly aphis swellings is a great increase in the number of tracheid fibers produced in the region affected. Approximately the same number of vessels is formed as in the normal stem, but the great increase in the number of tracheids makes it appear that fewer vessels are formed. Nothing was seen which indicated that burrknots ever arise from these woolly aphis swellings.

CONCLUSIONS

From the observations made in the study here reported and those made by other investigators it would seem that the formation of roots on stems has many points of similarity throughout the different plant groups in which it has been studied. Especially in apple and willow the same connection between root germs and the primary vascular system is found. Similar differences exist between different varieties of the same species in regard to the manner and amount of root formation (even apparently its entire absence in some varieties of both apple and willow). The chief differences between willow and apple in regard to the manner and amount of root formation are (1) speaking generally, the root germs on willow arise on younger wood and in greater numbers than those on apple, and (2) the apple root rudiments continue to elongate and to branch more than do those of the willow. With only these minor differences between apple and willow, it seems safe to conclude, in the absence of any contrary proof, either that in both apple and willow the formation of stem-borne roots is due to some pathogenic organism, or that no organism is concerned in either. The writer inclines to the latter view.

SUMMARY

This paper reports a histological study of burrknots in the apple and of the relation of their rudiments to the primary vascular system.

A celloidin-paraffin method of microtechnic is described whereby it was possible to cut, on the rotary microtome, complete series of 8 to 10 micron sections of 10-year-old apple stems.

A method is described of injecting the vascular bundles with dyes, which makes it possible to study in free-hand sections the course of the vascular strands in young apple stems.

The primary vascular system of the apple consists entirely of common bundles, each bundle connected with, i. e., ending above in, a leaf.

Each of the three bundles from every leaf passes down the stem a greater or less distance, ultimately merging with a bundle from another leaf. Before merging, each leaf trace may or may not divide into two or more parts. In some cases the two parts may run down on either side of a bundle entering from a lower leaf.

The general course of the bundles is straight downward in the stem, with few lateral connections. This fact would tend to explain in part the physiological results obtained by Auchter, who found little or no lateral diffusion of material in the apple stem.

The causes for the different courses taken by bundles of successive nodes in the same branch is unknown.

Root germs in the apple may be initiated at the following points in the cambium ring: (1) At the branch gaps, (2) at the leaf gaps, (3) at the primary medullary rays, and (4) at the secondary medullary rays.

The histological structure of woolly aphis galls on the apple is entirely different from that of burrknots.

The origin and arrangement of the root germs of the apple is very similar to the arrangement of root germs in willow, poplar, and other plants. It is concluded that similar physiological and genetic factors (rather than pathological factors) are involved in the formation of root germs in the apple and in the willow.

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FOUR NEW CHALCIDOID PARASITES OF THE PINE TIP MOTH, RHYACIONIA FRUSTRANA (COMSTOCK)[1]

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INTRODUCTION

A large number of parasites of the pine tip moth, *Rhyacionia frustrana* (Comstock), and its variety *bushnelli* Busck, have been reared in recent years by R. A. Cushman and other bureau workers. Among these parasites are four chalcidoids which appear to be new to science, and at the suggestion of Cushman, who is preparing a manuscript dealing with all of the parasites of this host, the following descriptions of these new chalcidoids are published.

FAMILY CHALCIDIDAE

Haltichella rhyacioniae, new species.

Very similar to *americana* (Howard), but may be distinguished from that species by the fact that the first tergite, in addition to being finely striated at extreme base, is finely shagreened over most of the remaining surface. In *americana* the first tergite is striated at extreme base, but otherwise perfectly smooth and polished. The new species differs from *longicornis* (Ashmead) and *onatas* (Walker) in that the scape and all legs are black. *H. xanticles* (Walker) is said to have the first tergite smooth and is considered by Ashmead to be the same as *americana*. *H. perpulchra* (Walsh) is said to have the abdomen glabrous, and the second tergite is apparently short. Types of *americana* and *longicornis* have been examined, but those of the Walker and Walsh species have not been seen.

Female.—Length, 3.60 mm. Head closely punctured all over and clothed with silvery pubescence, the interstices between punctures very finely shagreened; viewed from in front the head triangular; malar space only slightly shorter than the height of eye; antennae inserted at clypeus, nearly three times as long as the height of head, and very slightly clavate; scape long and cylindrical; pedicel about twice as long as thick at apex; third joint of antennae small, quadrate, or nearly so; fourth joint a little longer than second and third combined, about three times as long as thick; fifth to tenth successively decreasing slightly in length, the tenth about one and one-fourth times as long as broad; club ovate, longer than tenth joint but not as long as ninth and tenth combined; dorsum of thorax with pubescence and punctation like head, the punctures not quite as close and the shagreening of interstices a little less distinct; scutellum as long as prescutum, rounded at apex; propodeum gradually narrowing posteriorly, coarsely reticulated and rugulose, with four longitudinal carinae medially, the two nearest the middle strongest and inclosing a subfusiform area, which is three or four times as long as broad and extends from base to apex of propodeum, the two outer carinae curved or angulated and embracing a subcircular or octagonal area within which lies the median area already mentioned; laterad of the large octagonal area on the propodeum are three or four coarse reticulations and the whole surface of the propodeum is finely wrinkled; spiracles large and elongate; venation extending a little beyond middle of wing, marginal vein variable, about twice as long as stigmal; postmarginal very short; hind coxae and femora weakly shagreened and pilose, the femur about twice as long as broad, its ventral margin sinuate, with many very fine teeth; hind tibiae strongly curved, shagreened, its anterior side margined with a delicate carina from base to apex, calcaria two in number, short and unequal; hind basitarsus equal in length to the second tarsal joint; fore and median basitarsus much longer than second joint; abdomen conic-ovate, as long or a little longer than head and thorax; first tergite com-

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prising approximately one-third the dorsal length, almost devoid of hairs, finely striated on its basal one-third, weakly shagreened elsewhere, except a broad smooth border along the posterior margin; second tergite medially about two-thirds as long as first, pilose laterally, shagreened like the first, with the posterior margin and a large rounded area on each side smooth and devoid of hairs; following tergites all more or less shagreened and pilose; third about half as long as second; fourth and fifth short and subequal; sixth about as long as second; seventh as long as third; tip of ovipositor sheath exposed. Black; knees of front and middle legs, apices of all tibiae narrowly, and all tarsi dark reddish testaceous; antennae entirely black; wings hyaline, venation dark brownish.

Male.—Length, 2.80 mm. Antennal flagellum nearly three times as long as scape; scape as in female; pedicel barely longer than broad; flagellar joints thicker than in female; third antennal joint about two and one-half times as long as thick; fourth to tenth successively decreasing slightly in length, the tenth about one and one-half times as long as thick; club no thicker than tenth joint and about one-half longer; abdomen about as long as thorax behind the pronotum, sculptured as in the female, the first tergite usually comprising more than one-third the dorsal length, second about half as long as first, following short and subequal. Otherwise like the female.

Type locality.—Falls Church, Va.

Type.—Cat. No. 40178, U. S. N. M.

Host.—*Rhyacionia frustrana* (Comstock).

Type, allotype, 14 female paratypes, and 15 male paratypes reared in July, 1924, by R. A. Cushman from pupae of the above-named pine tip moth collected on *Pinus virginiana* at Falls Church, Va.; 18 paratypes, including both sexes, were reared in May, 1916, from the same host collected at Falls Church by Carl Heinrich (Hopkins U. S. No. 13924); 5 paratypes were reared from the same moth at Falls Church in 1925 by Cushman; 1 male paratype reared from the same host collected on Nantucket Island, Mass., by I. W. Bailey, in March, 1926. Fourteen paratypes were reared in July, 1924, by Jay Higgins from pupae of *Rhyacionia bushnelli* collected at Halsey, Nebr., and in July, 1925, 20 additional specimens were reared by L. G. Baumhofer from material of the same moth taken at the same place (Hopkins U. S. No. 17508).

FAMILY EULOPHIDAE

Hyssopus rhyacioniae, new species.

This is very close to *H. novus* (Girault), but may be distinguished from that species by its broader face and reddish testaceous hind tibiae. The eyes of *rhyacioniae* are also somewhat smaller than those of *novus* and not so conspicuously hairy, the hairs being shorter. Differs from *thymus* (Girault) in that the pronotum is more slender and the propodeum is without distinct median carina and lateral folds.

Female.—Length, 1.3 mm. Head shining, very faintly shagreened and sparsely clothed with grayish hairs; viewed from in front, very nearly circular in outline; antennae inserted near clypeus; frons a little wider than the vertical length of eye; scrobes moderately deep and subtriangular; eyes oval and thickly set with very short pile; mandibles with small teeth; viewed from above the head is transverse, as broad as thorax at tegulae and about twice as broad as long, the occiput hardly at all concave, temples rounded and immargined, ocelli in an obtuse triangle; postocellar and ocellocular lines subequal; antennae short, clavate; scape slender, not reaching to front ocellus; pedicel longer than thick, as long as first and second funicle joints plus ring joint; first funicle joint small, subtriangular, and about as long as broad at apex; second, third, and fourth funicle joints each about equal to the first in length but successively increasing in thickness, the fourth fully twice as broad as long; club short ovate, thicker than the last funicle joint and about as long as the three preceding funicle joints, practically solid, but with faint indications of a suture beyond the middle; pronotum cone-shaped, a little longer than broad at posterior margin, narrower than the mesonotum at tegulae, shining but weakly shagreened, the sculpture a little more distinct than on the head, sparsely clothed with rather long blackish hairs; mesothorax somewhat flattened dorsally, mesoscutum weakly shagreened,

parapsidal grooves sharply defined; scutellum a little more weakly shagreened than the prescutum and very slightly shorter than prescutum; prescutum with two pairs of black bristles, otherwise bare; scapulae each with one prominent bristle and also sparsely clothed with shorter hairs; scutellum bare except for two pairs of bristles located laterad of the longitudinal grooves; axillae bare; propodeum in nearly the same horizontal plane as the scutellum, practically smooth and shining, without median carina, lateral folds, or spiracular sulci; legs faintly shagreened and hairy; hind tibiae with two distinct but unequal calcaria; forewings reaching beyond the apex of abdomen; marginal and submarginal veins about equal, stigmal approximately one-fourth as long as marginal, postmarginal slightly longer than stigmal; discal ciliation moderately dense except at base of wing, which is mostly bare, but the vestigial median vein is marked by a very distinct row of discal cilia, beginning at base of wing and extending to the posterior margin a little beyond the middle of wing; the area behind this row of cilia is mostly bare except for an incomplete row along the posterior margin of wing and a few much weaker cilia forming an incomplete row between the two; the basal cell is bare; abdomen sessile, about as long as thorax, elliptical in outline, the first segment comprising approximately one-third its length, following segments subequal; venter with numerous bristles toward apex; ovipositor concealed. Black and shining, with a faint bronzy tint on the abdomen; all coxae black, femora black or piceous; all tibiae and tarsi dark reddish testaceous, the apical tarsal joint blackish; antennae piceous, the scape usually dark reddish testaceous; wings hyaline, venation brownish.

Male.—Indistinguishable from the female except by the genitalia and the fact that the abdomen is somewhat shorter and smaller than in the female.

Type locality.—East Falls Church, Va.

Type.—Cat. No. 40179, U.S.N.M.

Type female, allotype, and 17 paratypes reared by R. A. Cushman from larva of *Rhyacionia frustrana* (Comstock) during June and July, 1924.

Elachertus pini, new species.

Nearest to *proteoteratis* (Howard), but may be distinguished at once from that species and also from *coxalis* (Howard) by its black color. Differs from other described North American species by having an abdominal petiole that is fully as long as broad.

Female.—Length, 1.6 mm. Head, viewed anteriorly, distinctly broader than high; eyes prominent, hairy; malar space short; occiput very slightly concave, carinately margined just behind the ocelli but not elsewhere, distinctly shagreened; vertex and front very faintly shagreened; cheeks mostly polished; antennae inserted just above clypeus; scape cylindrical, slightly curved, almost reaching to front ocellus; pedicel longer than broad, a little shorter and not as thick as first funicle joint; first funicle joint slightly longer than broad; the second, third, and fourth funicle joints quadrate; club conic ovate, as long as two preceding funicle joints; pronotum conical but rather short, narrower posteriorly than the mesonotum at tegulae, distinctly shagreened all over and clothed with rather long brownish hairs; mesoscutum short, parapsidal grooves deep, scapulae prominent, prescutum much wider anteriorly than long down the middle; prescutum and scapulae shagreened and clothed with hairs about like pronotum; scutellum nearly twice as long as prescutum, finely and evenly shagreened or reticulated and bare between the grooves, laterad of grooves nearly smooth and with one long seta anteriorly and another posteriorly on each side; axillae weakly shagreened, not produced anteriorly into the posterior margin of mesoscutum but forming with the base of scutellum a nearly straight line; metanotum polished; propodeum with a strong median longitudinal carina, polished and bare medially, very faintly shagreened and clothed with a few long hairs laterad of the folds; marginal vein approximately two and one-half times as long as stigmal; postmarginal about two-thirds as long as marginal; abdominal petiole as long as broad; abdomen beyond petiole ovate, as broad as thorax at tegulae, and somewhat less than twice as long as broad; apex of ovipositor barely showing from above. General color black; vertex, frons, and especially the cheeks and oral region metallic green; thorax faintly tinged with aeneous; abdomen shining black with a pale yellowish spot basally; coxae black; femora mostly piceous; trochanters, tibiae, tarsi, and sometimes the femora at base, yellowish testaceous; wings hyaline.

Male.—Length, 1.4 mm. Funicle joints separated by a distinct short petiole and each clothed with hairs approximately as long as the segment; club terminat-

ing in a very short apical spine; abdomen somewhat shorter than in the female and more rounded at apex. Otherwise like the female.

Type locality.—Falls Church, Va.

Type.—Cat. No. 40180, U.S.N.M.

Five females (one type) and six males (one allotype) reared by R. A. Cushman from *Rhyacionia frustrana* in July, 1924. According to Cushman the species is a gregarious parasite of the larvae.

Secodella subopaca, new species.

In J. C. Crawford's key to species of *Secodella* [2] this runs best to *cushmani* and is very similar to that species, but more deeply sculptured on the mesoscutum and scutellum. In *cushmani* these sclerites are strongly reticulate-punctate, with a strong metallic green and somewhat brassy luster, while in the new species they are uniformly closely punctate and dull bluish green, with scarcely any luster.

Female.—Length, 1.9 mm. Head as broad as thorax, closely punctate and subopaque all over except bottom of antennal scrobe, which is shining; ocellocular line about equal to diameter of ocellus; antennal scape cylindrical, slightly curved; pedicel approximately one-third as long as scape, about one and one-half times as long as thick at apex; flagellum slightly clavate, the funicle joints successively increasing very slightly in width and the club barely wider than the last funicle joint; funicle joints subequal in length, the first slightly longer than pedicel and a trifle shorter than second funicle joint; club about as long as two preceding funicle joints combined, three-jointed, the two basal joints subequal and subquadrate; apical joint very short, conical, and terminating in a short spine; thorax about one-fourth longer than broad; pronotum short, closely punctate; mesoscutum closely and deeply punctate, dull; parapsidal grooves deep and sharply defined; axillae and scutellum sculptured exactly like the mesoscutum; propodeum short, somewhat shining, weakly shagreened laterally but more distinctly sculptured medially, the median longitudinal carina delicate but distinct, lateral folds and spiracular sulci absent; forewings reaching a little beyond apex of abdomen, the discal cilia arranged as usual for the genus but somewhat sparser and weaker than in some of the other species; marginal and submarginal veins nearly equal, postmarginal about one-fifth as long as marginal and one and one-half times the stigmal; hind coxae outwardly rather strongly punctate; abdomen a little longer than head and thorax, distinctly narrower than the thorax, its sides parallel except the apical one-fourth, which is conical; first tergite smooth and constituting about one-fourth the length of abdomen; following tergites all finely shagreened; second, third, and fourth tergites subequal in length, the fifth somewhat longer than the fourth and the sixth still longer than the fifth; apex of ovipositor barely exserted. Head dull bluish in color with a faint tinge of greenish; antennal scape and pedicel concolorous with the head, the flagellum black but covered with dense grayish pubescence; thorax dull blue-green, the pleura purplish in some lights; propodeum and first tergite shining metallic green; tergites beyond the first darker, with a faint metallic sheen; abdomen beneath strongly purplish; wings hyaline, venation brownish; legs concolorous with the thorax except that the basal three joints of middle and hind tarsi are pale and the front tarsi fuscous.

Male.—Length, 1.25 mm. Antennal scape flattened, a little more than twice as long as broad; club not thicker than the funicle, three-jointed, the apical joint terminating in a spine; head and thorax not quite so deeply sculptured as in the female and therefore appearing slightly more shining; abdomen not longer than the thorax, compressed from the sides. Otherwise agrees with the female.

Type locality.—Falls Church, Va.

Type.—Cat. No. 40181, U.S.N.M.

Type female and five female paratypes reared by R. A. Cushman from *Rhyacionia frustrana* (Comstock) collected in the vicinity of Falls Church, Va., in June and July, 1924. The allotype male was reared by Cushman April 17, 1925, from the same host collected at Bogalusa, La., by R. A. St. George.

One female paratype is slide mounted, the others are on card points. The antenna of the allotype is mounted on a slide.

[2] CRAWFORD, J. C. THE GENUS SECODELLA IN NORTH AMERICA. Ent. Soc. Wash. Proc. 17: 142. 1915.

THE CRITICAL TEMPERATURE OF THE CHICKEN[1]

By H. H. MITCHELL and W. T. HAINES, *Illinois Agricultural Experiment Station*

INTRODUCTION

In the course of a somewhat extensive series of investigations of the energy metabolism of chickens and of the net-energy value of feeds for chickens, it became necessary to determine the critical temperature of this animal—that is, the environmental temperature at and below which the heat production of the fasting resting animal will increase to prevent a lowering of body temperature.

REVIEW OF LITERATURE

The first reported investigation of the critical temperature of the chicken appears to be that of Regnault and Reiset, published in 1850 (*10*)[2]. These results, recomputed by Gerhartz (*3*), indicate a continuous decrease in the oxygen consumption of fasting hens as the environmental temperature increases from 19° to 23° C. However, there appears to be no assurance that the activity of the birds was sufficiently controlled in these experiments, a requisite to investigations of energy metabolism the importance of which was not appreciated at this early date.

Gerhartz himself (*3*) has published respiration data from two hens exposed in a fasting condition to external temperatures ranging from 21° to 32° C. The computed oxygen consumption per kilogram body weight per hour, when plotted against temperature, indicated a sharp drop in metabolism at about 23°, followed by a rapid rise at lower temperatures. This temperature was considered to be the critical temperature of the hen. There are, however, several objections to this interpretation. The hens had fasted for only 13 to 24 hours. In the writers' experiments it was found that the effect of food on heat production may extend over a much longer period. Hence, Gerhartz's hens can not be considered as having been in the postabsorptive condition, a condition obviously necessary to a determination of the critical temperature. Furthermore, the sharp rise in oxygen consumption shown by Gerhartz's curve for temperatures lower than 23° may be an effect of individuality rather than of temperature, since the values for 23° and above were obtained from hen 1, while the values below 23° were obtained from hen 2. It is questionable whether values obtained from two animals at distinct temperature ranges can be safely compared in this way.

[1] Received for publication Jan. 11, 1927; issued May, 1927.
[2] Reference is made by number (italic) to "Literature cited," p. 557.

Later investigations on the energy metabolism of geese (*6*), ducks (*7*), and other birds (*4*), aside from indicating more or less certainly a stimulating effect of low temperatures on heat production, have not contributed any definite information as to the critical temperatures of birds.

METHODS AND APPARATUS

In the present study, the respiratory exchange of the hens was determined by means of the gravimetric method of Haldane (*5*). By this method, open circuit in principle, the animal chamber is ventilated with a stream of air that has been rendered dry and free of carbon dioxide by passage through sulphuric acid and caustic alkali (or soda lime). The outgoing air is again passed through these reagents in appropriate containers. The carbon dioxide pro-

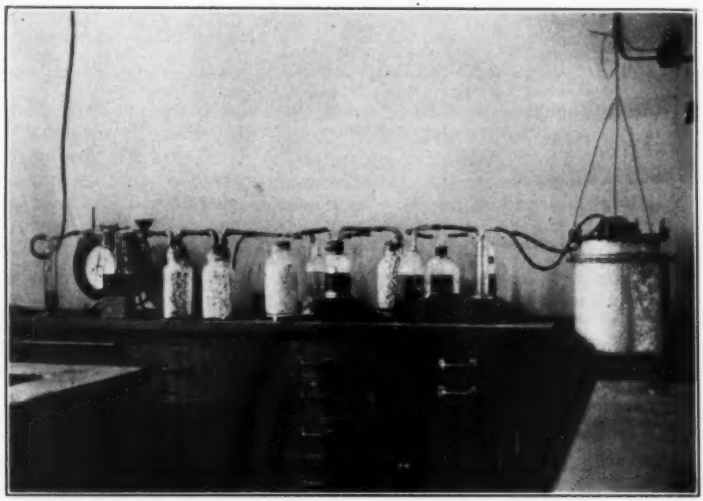

FIG. 1.—Apparatus used in critical temperature studies

duction of the animal is measured by the increase in weight of the caustic alkali bottle and of the sulphuric acid container following, while the oxygen consumption is measured by the total increase in weight of the animal chamber and the entire outgoing chain of bottles. An apparatus such as was used by the writers in much of their work with chickens is shown in Figure 1.

Since this particular investigation deals entirely with fasting birds, in which, according to the writers' observation, the respiratory quotient is fairly constant at 0.70 to 0.73, the oxygen determination was dispensed with and variations in heat production were measured by variations in the production of carbon dioxide. The outgoing chain consisted of a Williams bottle next to the chamber, for the removal of water, and a small bottle filled with caustic soda ("shell caustic"), followed by a small gas-washing bottle containing sulphuric acid.

The two last-mentioned containers were much smaller than those illustrated, since the experiments were less than one hour in duration. The smaller size permitted more accurate weighing. Between the Williams bottle and the caustic-soda bottle was a two-way stopcock leading either to the caustic-soda bottle or to the pump directly by way of a rubber tube. By manipulation of this stopcock, the outgoing air could be drawn through the carbon dioxide absorption chain or could be diverted from it.

The experiments were conducted according to the following general plan: All the hens were allowed to fast for at least 41 to 48 hours, since, according to the writers' observation, this length of time is required for the postabsorptive condition to be reached after a normal meal. To avoid any possibility of error, most of the hens were allowed to fast for 72 hours. As soon as a hen was placed in the animal chamber the chamber was sealed with paraffin, ventilation was started, and the stopcock on the outgoing chain was so turned that the air passed directly to the pump. The animal chamber was then brought to the desired temperature by exposing it to a draft of cold air, aided if necessary by the placing of ice on the top of the chamber. The temperature of the air as it emerged from the chamber was taken as the environmental temperature of the hen. The collection of carbon dioxide was not started until the hen had been exposed for at least 30 minutes to the experimental temperature.

In such an investigation as this it is essential to the proper interpretation of the results that the activity of the hen shall be at the lowest possible minimum. This was assured in two ways. The chamber used was just large enough to contain the hen snugly. Any considerable moving around was thus rendered impossible. Furthermore, the hen was in complete darkness, a condition known to favor quietness in hens. For the detection of small movements of the hen, the animal chamber was suspended from a spring. (Fig. 1.) By noting the up and down movement of the chamber, or, better yet, by placing the hand beneath it, slight movements of the hen could be detected. In all of the experiments, the hens were completely at rest, in so far as this condition could be determined by the method used. If, after the air was permitted to pass into the carbon dioxide absorption bottle, any appreciable movement of the hen was noted, the air was immediately diverted to the pump, and was not again admitted to the caustic soda bottle for at least five minutes after the hen had come to complete rest. The time was noted at each turning of the stopcock, in order to obtain the total time of collection, and the rate of ventilation, as measured by a gas meter, was kept constant throughout the period of observation at approximately 3 liters per minute. With these precautions, it is believed that the results obtained possess a definite significance.

EXPERIMENTAL DATA

Twelve Rhode Island Red hens, ranging in weight from about 4 pounds to over 6 pounds, were used in this investigation. Thirty-six observations were made upon these hens, with the results shown in Table 1.

TABLE 1.—*The carbon dioxide production of hens at different external temperatures*

Experiment No.	Hen No.	Date of test	Hours of fast	Body weight	Carbon dioxide production per minute at temperatures of—								
					85° F.	80° F.	75° F.	70° F.	65° F.	60° F.	55° F.	50° F.	45° F.
		1925		*Gm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	2429	Jan. 2	41	2,026			26.8	27.0	23.6	24.0	26.0		
2	2158	Jan. 5	46	2,325				22.5	24.4	30.1	36.0		
3	2420	Jan. 6	46	2,717				26.1	23.1	28.8	30.9		
4	2089	Jan. 7	48	2,500			25.4	26.0	24.8	24.0			
5	2143	Jan. 8	48	2,997			29.1	24.2		24.0			
6	2117	Jan. 9	48	2,233				21.4	21.9	25.6	27.9		
7	2429	Jan. 10	48	1,927					21.9		22.9		
8	2084	Jan. 12	72	2,086			24.5	20.9	23.0				
9	2058	Jan. 14	72	2,509		25.1	30.7						
10	2053	Jan. 15	72	2,710			24.5	27.4	25.9	24.0			
11	2013	Jan. 16	72	2,665			27.2	23.3	28.5		30.0		
12	2000	Jan. 19	72	1,850			19.2	13.8	21.3		22.9		
13	2009	Jan. 20	72	2,485			20.0				24.8		
							19.0				22.4		
14	2158	Jan. 21	72	2,412			25.0				26.5		
							24.8				30.0		
15	2420	Jan. 22	72	2,788			20.0		22.5				
							17.0		25.4				
16	2089	Jan. 23	72	2,625			22.8		20.5				
							21.4		22.0				
17	2143	Jan. 26	72	3,017			24.4		24.2				
							25.9						
18	2117	Jan. 27	72	2,347			17.6		21.1				
							17.5		14.4				
19	2084	Jan. 28	72	2,220			21.7		24.2				
									20.5				
20	2058	Jan. 29	72	2,595			27.8		30.0				
							22.1						
21	2053	Jan. 30	72	2,717			27.2		31.5				
									30.2				
22	2013	Feb. 2	72	2,749					20.5		35.1		
									24.4		27.3		
23	2420	Feb. 3	72	2,809							22.0		24.0
											21.6		27.0
											22.0		
24	2000	Feb. 4	72	1,905							24.8		26.9
											20.5		27.5
25	2009	Feb. 5	72	2,562	20.7		24.7						
					20.0		22.7						
26	2158	Feb. 6	72	2,657	24.3		21.1						
					21.0		21.2						
							22.5						
27	2429	Feb. 9	72	2,145	27.5		18.7						
					23.0		18.4						
							19.5						
28	2089	Feb. 10	72	2,685				24.4		29.2			
								19.2		28.8			
29	2143	Feb. 11	72	3,042					30.6		30.7		
									28.0		35.8		
30	2117	Feb. 12	72	2,430						17.4		24.8	
										19.2		24.0	
31	2084	Feb. 13	72	2,387						29.8		26.1	
										26.4		29.5	
32	2058	Feb. 16	72	2,745						24.3	29.7	37.0	
										27.7			
33	2053	Feb. 17	72	2,875						30.0		30.4	
										28.5		35.3	
34	2013	Feb. 18	72	2,970						26.6		26.9	
										25.9		31.4	
35	2420	Feb. 19	72	2,940						24.7		26.4	
										22.7		27.2	
36	2000	Feb. 20	72	2,062					21.8	24.7		28.1	

Environmental temperatures ranging from 45° to 85° F. were imposed, and in many of the experiments duplicate or triplicate determinations of the carbon dioxide production were made at each of the temperatures used. The order in which the different temperatures were imposed is not indicated in the table, since, from a study of the records, it did not appear to be a matter of consequence.

Evidently the individual experiments were not planned—at least in most cases—in such a way that the exact location of the critical

temperature could be determined. The most that any one experiment indicates is that the desired temperature is above or below a given point. Therefore in their most complete interpretation, they must be considered in the aggregate. The experiments have been summarized in Table 2, according to the following scheme:

TABLE 2.—*The number of experiments which indicate the location of the critical temperature either above or below the environmental temperatures imposed*

Temperature imposed	Number of experiments locating the critical temperature—	
	Below the temperature imposed	Above the temperature imposed
°F.		
45	0	23
50	1	21
55	3	17
60	6	12
65	12	8
70	13	2
75	14	2

1. If the results in experiments involving only two temperatures indicate, more or less certainly, that the production of carbon dioxide per minute was greater at the lower than at the higher temperature, they are interpreted to mean that the critical temperature is above the lowest of the two temperatures. Its location with reference to the highest temperature can not be inferred. In experiment 13, for example, the two observations at 75° F., i. e., 19 and 20 mgm., are lower than those at 55°, i. e., 24.8 and 22.4 mgm. Hence, the critical temperature is evidently above 55°, but whether it is above or below 75° the results do not indicate. Since, in this experiment, the critical temperature is above 55°, it must also be above 50° and 45° and is so listed in Table 2, where it is entered at these three temperatures.

2. Similarly, if a series of continuous observations extending over more than two temperatures indicate a continuous increase in carbon dioxide production with each decrease in temperature, the conclusion is justified that the critical temperature is above the second highest temperature of the series. Experiment 2 is a good example of this type. A continuous increase in carbon dioxide production was observed from 70° to 55° F. It is evident that the critical temperature in this experiment is above 65°, but whether it is above or below 70° can not be decided, since the increase in carbon dioxide production observed may have started either at a point between 65° and 70° or at some point above 70°. In this case, therefore, it can only be concluded that the critical temperature is above 65°, and hence above all lower temperatures. In Table 2, therefore, this experiment is listed as indicating a critical temperature above 65°, 60°, 55°, 50°, and 45°, thus entering the table in five places.

3. If a series of two or more continuous observations at different temperatures shows no significant effect of environmental temperature on carbon dioxide production, the conclusion is justified that the critical temperature is below the lowest temperature of those at which observations were taken. An illustration of such an experiment is afforded by experiment 10, which indicates a critical temperature below 60° F., experiment 16, which indicates a critical

temperature below 65°, and experiment 31, which indicates a critical temperature below 50°. Experiment 10 was used in the construction of Table 2 as indicating a critical temperature below 75°, 70°, 65°, and 60°; experiment 16, as indicating a critical temperature below 65°, and all higher temperatures; and experiment 31, as indicating a critical temperature below 50° and all higher temperatures.

4. If, in a series of observations extending over several temperatures, it appears that the carbon dioxide production of the hen increases somewhere between two of the temperatures chosen, it is inferred that the critical temperature is similarly located. Experiment 6 represents such a case, and in the construction of Table 2 is taken as indicating a critical temperature above 60°, 55°, 50°, and 45°, and below 75°, 70°, and 65°, thus entering the table at seven places. Experiment 11 is similar.

5. In a few of the experiments, a tendency to an increase in carbon dioxide production at the higher temperatures is evident. Since such an increase bears no relation to the critical temperature, it is not considered in the construction of Table 2. Such an increase may be noted in experiments 5, 11, and 27.

Having considered the method used in summarizing and evaluating the results in Table 1, the results given in Table 2 may now be studied with the idea of obtaining an average value for the critical temperature of the chicken. None of the experiments indicated a critical temperature below 45° F., while 23 indicated one above this temperature. For 50°, one experiment pointed to a critical temperature below, while 21 pointed to a critical temperature above. The figures in the two columns at the right approach equality between 60° and 65°. For observations taken above 65°, the evidence points unmistakably to the conclusion that the critical temperature is below this range rather than above. The evidence as a whole, therefore, may be taken to point to an average critical temperature for the Rhode Island Red hen of approximately 62° F. or 16.5° C. Since these birds were taken from the university poultry farm in the middle of winter, this critical temperature may be considered as applying to chickens under winter conditions of feathering and under the condition of low humidity obtaining in the animal chamber.

This average critical temperature of 16.5° C. for the winter-feathered Rhode Island Red hen may be compared with the critical temperature of about 21° C. for the pig, as recently determined by Capstick and Wood (*1*). Forbes and his associates (*2*) have reported a critical temperature greater than 18.3° C. for a steer with closely shorn hair and less than 15.5° for a steer carrying a full coat of hair. Morgulis (*9*) determined the critical temperature of a female Irish terrier before and after clipping to be, respectively, between 13.6° and 15.1°, and between 23.8° and 26.5° C. The critical temperature of the goat, determined by Magee, (*8*) is not comparable to the values above given, since the animal was not fasting but was receiving approximately a maintenance ration. Under this condition, the critical temperature appeared to lie between 12.7° and 21.1° C. In the fasting state the temperature would be higher still.

A study of the individual reactions of the 12 hens used in the metabolism experiments herein reported reveals considerable differences. The best judgment of the writers as to the location of the critical temperatures of the individual birds is given in Table 3.

TABLE 3.—*Probable location of the critical temperatures of the individual hens*

Hen No.	Number of experiments	Probable location of critical temperature (° F.)
2429	2	Below 55.
2158	3	Between 65 and 75.
2420	4	Above 65.
2089	3	Inconclusive.
2058	3	Above 75.
2013	3	Between 65 and 75.
2000	3	Above 65.
2009	2	Above 75.
2084	3	Below 50.
2143	3	Between 55 and 60.
2117	3	Between 60 and 65.
2053	3	Inconclusive.

The reaction of hen 2084 stands in marked contrast to that of hens 2058 and 2009. Such differences may be due to differences in feather covering or in the amount of subcutaneous fat, or in the vasomotor control of the birds. They represent the penalty of working with several animals rather than with one, in so far as the consistency of the data obtained is concerned.

Although the practical importance of the location of the critical temperature of the fasting farm animal is obvious, it should be realized that it is subject to variations, not only in the animal itself, but in its thermal environment. At constant low temperatures the heat production of an animal may vary with the humidity of the surrounding atmosphere or with the prevalence and intensity of air currents. It is the cooling power of the surrounding air, rather than its temperature alone, that determines whether the heat produced in metabolism is sufficient to maintain body temperature. Investigations concerned with the relative importance of temperature, humidity, and air movement would be of great interest and value.

RATE OF INCREASE IN HEAT PRODUCTION OF ANIMALS EXPOSED TO ENVIRONMENTAL TEMPERATURES BELOW THE CRITICAL

Another problem of importance in this connection relates to the rate at which the heat production of an animal will increase per degree fall in the environmental temperature below the critical. It is known that above the critical temperature the emission of heat from the body surface is regulated by vasomotor reflexes, by reflex stimulation of the sweat glands, and at high temperatures by the type of breathing. If it may be assumed that at the critical temperature the radiating capacity of the skin is reduced to the lowest possible minimum by vasoconstriction of the arterioles feeding the subcutaneous capillary bed, and that the secretory activity of the sweat glands has been totally inhibited, it is reasonable to suppose that Newton's law of cooling bodies will apply, and that

$$H = k\ (t - t^1),$$

where H is the heat emission (or heat production) at the critical temperature, t is the normal body temperature of the animal, t^1 the critical environmental temperature, and k a constant equal to the increase in heat emission (or heat production) per degree drop in temperature. If this equation represents the conditions prevailing,

the increase in heat production with decrease in temperature below the critical should be linear.

The only experimental data that appear to be available for testing the applicability of this equation to animals in environments below the critical are those of Capstick and Wood on the pig.[3] These data indicate a linear increase in heat production with decreasing environmental temperatures below the critical, as the equation would predict. The experimental data also indicate that the increase occurred, in the particular pig observed, at the rate of 0.077 calorie per minute per degree drop (*1, p. 265*). It is interesting to compute the rate of increase predictable from the above equation. At 20.4° C. (the approximate critical temperature), the basal heat production of the pig, corrected to a weight of 300 pounds, was 1.499 calories per minute. If the body temperature of the pig be taken as 39.5° C. (*11, p. 90*), the equation becomes

$$1.499 = k\ (39.5 - 20.4)$$

$$k = 0.078 \text{ calorie.}$$

This value of k, computed from a theoretical consideration of the conditions prevailing at the critical temperature, is in remarkably close agreement with the value experimentally determined (0.077 calorie) from observations at and below the critical temperature. This agreement, together with the linear character of the rise observed in the heat production as the temperature fell below the critical, may be taken as legitimate confirmation of the applicability of Newton's law of cooling bodies to the relation between the basal heat production of the pig and the environmental temperatures at and below the critical.

If the formula may be assumed to apply to the hen also the practical significance of the value of k may be illustrated as follows:

A 5-pound hen, according to unpublished data, will possess an average basal heat production of approximately 115 calories per day. If the body temperature of the hen be taken as 106° F. and the critical temperature as 62° Newton's formula becomes

$$115 = k\ (106 - 62), \text{ and}$$

$$k = 2.6 \text{ calories per degree Fahrenheit.}$$

This means that for each drop of 1° F. in the temperature of the environment below the critical, the heat production of the hen must increase 2.6 calories per day in order to maintain her body temperature. If the hen is moving around actively in a small pen, the writers' data indicate that her heat production may easily be increased by 70 calories daily; the critical temperature of the fasting *active* hen may be computed to be $62 - (70 \div 2.6) = 35°$. If the hen is consuming a ration composed largely of corn, sufficient in amount to support an egg production of one per day (estimated to be 0.217 pound), the heating effect of this amount of food may be estimated, from unpublished data, at 51 calories. This amount of heat is sufficient to cause a further drop in critical temperature of about 20°. Therefore, it may be roughly estimated by the use of Newton's con-

[3] In no one of the experiments reported in Table 1 was the critical temperature of the hen located with sufficient definiteness to permit its use in this sort of a calculation.

stant that a 5-pound hen confined to a small pen and receiving a sufficient ration, based mainly on corn, to support the production of one egg a day, could stand an outdoor temperature of 15° before requiring additional food to keep her body warm. Evidently this rough prediction would be too low if the atmosphere were excessively humid, increasing the heat loss from the body by conduction, or if the hen were exposed to a brisk wind, which would have the same effect.

SUMMARY

From 36 experiments with 12 Rhode Island Red hens, involving 137 determinations of the carbon dioxide production during fast and quiescence at different temperatures, it was found that the average critical temperature was 62° F. This value applies to winter-feathered birds in an atmosphere of low humidity and in an air current of approximately 3 liters per minute. Some of the individual birds appeared to exhibit distinct differences in their reaction to changes in environmental temperature.

It is shown that Newton's law of cooling bodies may apply to animals at environmental temperatures below the critical. The practical significance of this conclusion is discussed and illustrated.

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HEREDITARY AND ENVIRONMENTAL FACTORS THAT PRODUCE MOTTLING IN SOY BEANS[1]

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INTRODUCTION

Mottling in soy beans has been considered as an inherited character and one that indicates genetic impurity. For this reason all soy-bean seeds blotched with irregular patches of black or brown pigment have been regarded as objectionable by most farmers and seed dealers. For the same reason the International Crop Improvement Association has recommended that all mottled soy beans be looked upon as "mixed," that they be ruled out of "registered seed," and that they be tolerated to the extent of only 1 per cent in "certified seed."

So pronounced has been the unpopularity of mottled soy beans that many efforts have been made to eliminate mottling by breeding and selection. In some instances these efforts seem to have been successful, but in certain sections of the country mottling has persisted in spite of all attempts to overcome it. This fact has been surprising to those familiar with the uniformity of colorless or non-mottled varieties of the common bean (*Phaseolus vulgaris*), and the cause of the peculiar phenomenon of mottling in soy beans has puzzled technical investigators as well as practical growers.

The purpose of the present study was to determine how far one is justified in discriminating against mottled soy beans and to ascertain if possible the factors which cause the mottling. There is much yet to be learned concerning the fundamental physiological processes involved in mottling, but the results so far obtained show that environment as well as heredity plays a very important rôle in the process. This knowledge may be of some value to those who are concerned with the practical bearing of the question, and it is also hoped that the observations herein reported will stimulate others to further research.

OCCURRENCE OF MOTTLING AND THE WORK OF PREVIOUS INVESTIGATORS

In the United States the problem of mottling has been discussed in meetings of the National Soy Bean Growers' Association, and work from a number of stations has been presented. At the meeting of this association in September, 1925, J. B. Park, of the Ohio Experi-

[1] Received for publication Dec. 13, 1926; issued May, 1927. Contribution from the Department of Genetics (paper No. 66) and the Department of Agronomy, Wisconsin Agricultural Experiment Station. Published with the approval of the director.

The work on mottling was begun at the Wisconsin station in 1922 by B. D. Leith and V. A. Tiedjens. The present writer took over the problem in 1923 in connection with a study of the genetic factors involved in seed-coat color inheritance. In conducting the studies the writer was greatly aided by the cooperation of Professor Leith, of the Department of Agronomy, and Profs. L. J. Cole and R. A. Brink, of the Department of Genetics. Dr. E. J. Kraus, of the Department of Botany, has also given many valuable suggestions and C. R. Burnham kindly read and criticised the manuscript. It is a pleasure to acknowledge the advice and constructive criticisms which have been received.

[2] Now with the Maine Agricultural Experiment Station.

ment Station, gave a summary of the work reported on mottling up to that time. He referred chiefly to the graduate work of J. S. Culter at Ohio State University and of E. A. Hollowell at Iowa State College. Park emphasized the fact that whereas mottling has been found to be of very common occurrence in some parts of the country, in other sections it is almost unknown.

Hollowell's thesis [3] covers the results of three years' work. From tests which he supervised in various parts of Iowa he concludes that mottling may be greatly affected by soil conditions but not by climatic conditions. He found that more mottling occurred on rich soils than on soils deficient in fertility and that plants grown close together produced less mottling than plants spaced some distance apart in the row. Hollowell's work has much in common with that carried on by the present writer at Wisconsin.

In the Orient, where the soy bean has long been an important crop, no particular study of mottling has ever been made so far as the writer is aware, but from all reports mottling is also of common occurrence there. Nagai (*8*, *9*)[4] has done considerable work in Japan on the inheritance and chemistry of soy-bean pigments, but his studies will be referred to in a later part of this paper.[5]

PIGMENTS WHICH PRODUCE MOTTLING

Two pigments are primarily concerned with the production of seed-coat colors in soy beans, namely, black and brown. In black seed coats there is frequently considerable brown present in the spongy parenchyma cells (pl. 1) and in certain cases there is undoubtedly a mixture of the two pigments in the Malpighian cells. In mottled seed coats the pigments are the same as in those of self-colored varieties, the only difference being the complete development of the pigment to produce the self colors and the restriction of pigment in the mottled types. Data which are not entirely conclusive show that the seed coats of black or brown varieties are heavier than those which are free from these pigments, indicating that the pigments have an appreciable weight. No significant difference, however, has been found between the weight of mottled and nonmottled seed coats.

The black pigment is bright red for some time before the seed matures. Then as the seed becomes desiccated the color changes to purple and gradually becomes intensified. The brown pigment first appears as very light pink, but this is well toward the period of maturity, and the typical brown color is not apparent until the seed is ripe.

The black, or deep purple, pigment turns red in acid solution, and has all the general properties of anthocyanins. It is precipitated by salts of heavy metals and turns brown or green in alkaline solution, the exact shade depending on the alkali used. Hydrogen peroxide or sodium sulphite decolorizes a solution of the pigment very rapidly. It is very slightly soluble in pure water and almost entirely insoluble in 95 per cent alcohol, but either of these solutions becomes a fairly good solvent when made slightly acid. A 50 per cent solution of

[3] HOLLOWELL, E. A. FACTORS INFLUENCING THE MOTTLING OF THE SOY BEAN SEED COAT. 1924. [Unpublished master's thesis. Copy on file, library, Iowa State College, Ames.]

[4] Reference is made by number (italic) to "Literature cited," p. 586.

[5] The writer is especially indebted to Prof. Isaburo Nagai for answering correspondence relative to the mottling problem and to other investigators throughout China and Japan who have also answered inquiries and have sent samples of seed.

alcohol containing 1 per cent hydrochloric acid is an excellent solvent. Acetone is also a good solvent, especially when slightly acidified, but propyl and butyl alcohols are rather poor solvents for extracting the pigments from the tissue of the seed coat.

Except for a difference in color, most of the chemical reactions of the brown pigment are very similar to those of the black. Nagai (*8*) has used the term "phlobaphene" in connection with this brown pigment, as well as with the brown pigments which occur in many other plants. He found that this term dates back to Stähelin and Hofstetter in 1844, but it has been a general term applied to brown pigments known to be derived from tannins. The exact chemical nature of most of these substances is not known, but since Sando and Bartlett (*16*) have identified an isomer of quercetin in the brown husks of Emerson's maize it would not be surprising to find that many other brown pigments are also flavone glucosides.

Neither the black nor the brown pigment is soluble in ether, chloroform, carbon disulphide, or similar solvents; and ether precipitates them when added in excess to an alcoholic solution. This should provide a means of purification, but so far the writer has not been successful in getting either pigment in a pure crystalline form. One difficulty is perhaps due to the fact that there is a mixture of the two pigments in black seed coats. It is also possible that various types of flavone and flavonol glucosides may be present as mixtures.

It is doubtful whether there are but two pigments primarily concerned in seed-coat colors of soy beans, yet the situation seems much simpler than with many other legumes. In species of Phaseolus the color types are much more complicated, and while anthocyanin-like pigments probably cause most of the coloration, there must be numerous modifications to produce the various shades of reds, browns, and purples that are found. In the cowpea, Mann (*6*) found that black and brown melanin pigments occur as well as anthocyanin; and, in general, the seed coats of legumes are well known for their diversity of color types and patterns.

Besides the regular black and brown pigments in the seed coats of soy beans, there is an oxidation pigment which is frequently associated with the seed coats of black-podded varieties. Sometimes it produces a smudgy appearance, especially when the pod and testa are injured, but it is interesting to note that this pigment is localized chiefly in the parenchymatouslike cells of the seed coat, while the common black and brown pigments are largely restricted to the outer Malpighian cells. Since this pigment is not related to the brown and black pigments, however, no further mention will be made of its occurrence.

Another type of pigment has also been observed by Matsumoto and Tomoyasu (*7*) which is caused by a fungus (*Cercosporina kikuchii*) that is parasitic in the seed coat of the soy bean. Considerable purple mottling and seed injury are reported in certain instances as a result of the action of this organism.

FACTORS KNOWN TO INFLUENCE THE DEVELOPMENT OF ANTHOCYANIN PIGMENTS

Onslow (*11*) concludes that anthocyanin pigments are very widely distributed in the plant kingdom, and the species that do not become colored to some extent under suitable conditions are rare. Certain

environmental conditions are known to favor the production of pigment, and if these are not provided the pigment-producing potentialities of a plant may not become apparent. Since the anthocyanin pigment in the seed coat of the soy bean is very similar in chemical behavior to anthocyanins which occur in other plants, considerable may be learned from a consideration of the factors which are known to favor the production of these pigments.

Among the factors other than heredity that are responsible for the development of anthocyanin, light, temperature, oxygen supply, drought, and altitude have all been elaborated upon, but the most important fundamental factor seems to be the accumulation of synthetic products, especially sugars.

MacGillivray,[6] working with tomato plants, found that pigmentation was intimately associated with the phosphorous supply. When phosphorus was not available there was a very noticeable decrease in the production of nitrogenous compounds and this caused his plants to become highly colored. Under such conditions it seems natural that plants should be high in carbohydrates, and analyses showed this to be the case.

Since anthocyanins are glucosides, one would expect carbohydrates to be very essential for their formation; and Onslow's (*10*, *11*) work shows that the form in which the carbohydrates occur is also important. Overton (*12*), Katić (*4*), and Klebs (*5*) previously found that certain species of plants develop pigment very quickly when fed on sugar solutions. Cuttings of land plants were used in their experiments as well as water plants. Onslow (*10*), continuing this study, found that after she had fed a 3 per cent cane-sugar solution for 7 to 10 days, certain species of land plants developed pigment and others did not. A test for starch was then made on all the species under observation. Again it was found that some species formed starch and others did not. The interesting point in this connection is the high negative correlation that was found between starch and pigment formation. In species that were not able to synthesize starch, sugars accumulated; and since sugars are used in the formation of anthocyanins, a logical reason can be given for pigmentation. Plants that were able to form starch, she assumes, made use of their sugars in that process, and the quantity of sugar left was insufficient for the production of pigments.

A great deal of space would be required to review the observations published on the relation between the accumulation of sugars and pigment formation. Suffice it to say that the physiological condition of the plant is apparently the ultimate determining factor. In many cases the problem becomes very complicated. Especially is it difficult to distinguish factors that are directly concerned with pigment formation from those that are only indirectly concerned in that they affect the food supply of the plant.

Two of the outstanding factors which have been studied are light and temperature. Light is very probably a critical factor in the direct chemical processes that bring about certain anthocyanin pigments. Temperature also seems to be an important factor in certain instances, but it is difficult to distinguish its direct influence on pigment formation from the indirect influence which it exerts through

[6] MacGillivray, J. H. Effect of phosphorus on the nutrition of the tomato plant. 1925. Unpublished doctor's thesis. Copy on file, library, Univ. Wisconsin, Madison.]

its effect on the accumulation of food material in the plant. For a more detailed treatise on this subject the reader is referred to Onslow's (*11*) "Anthocyanin Pigments of Plants," which also contains an excellent bibliography.

ENVIRONMENTAL FACTORS IN RELATION TO MOTTLING

To find the determining factors in the production of mottling, limited studies have been made of plants grown under artificial conditions, but the most striking results have been obtained by carefully observing the behavior of plants in the field. These observations have confirmed the conclusion of Hollowell [7] that rich soils and a liberal distance between plants are usually conducive to mottling. Different soils have produced striking differences in the production of mottling, but the variability on the same type of soil and even on the same plant has also thrown much light on the cause of mottling. These studies have given conclusive evidence that environment affects the production of mottling and also that a delicate nutritional balance exists which likewise influences it.

Growing plants under different conditions and studying the variations which occur have contributed much information to the general problem, but the most valuable findings have been obtained from certain abnormalities.

DISTRIBUTION OF MOTTLED SEED ON THE PLANT

Woodworth and Cole (*18*) found that the distribution of mottled soy-bean seed may be extremely variable. They observed striking resemblances among seeds borne on the same plant, but breeding results did not indicate that these were due to heredity. They concluded that physiological factors must have been responsible for the results obtained.

In this discussion it may be well to remind the reader that the pigments under consideration are formed in maternal tissue. The portion of the seed coat in which the pigment occurs is derived from the outer integument of the ovule and is independent of the embryo, which belongs to a new generation. Hybridization, of course, may affect the embryo immediately, but the seed coat begins to form before fertilization, and for this reason all the seed coats of any one plant should be genetically identical, no matter how much hybridization has taken place. For this reason hybrid seeds can not be detected by any difference in the seed coat until the seeds are grown and another generation of seed coats is produced. If a pollen grain from a black variety of soy bean chances to fertilize an ovule from a brown variety the hybrid seed will still be brown and can not be distinguished on the basis of seed-coat color from any of the other seeds borne by the same plant; but when the hybrid seeds are grown and produce F_1 plants all the seeds borne by one of these plants will be black instead of brown. In this way the seed coat is always one generation behind the cotyledons because the cotyledons are part of the new embryo and may be immediately affected by hybridization.

Since all the seed coats on one plant are made up of cells which supposedly have the same hereditary potentialities, one would

[7] HOLLOWELL, E. A. FACTORS INFLUENCING THE MOTTLING OF THE SOYBEAN SEED COAT. 1924. [Unpublished master's thesis. Copy on file, library, Iowa State College, Ames.

expect all to be the same color or pattern if these depended entirely on the hereditary constitution. If the variation in mottling were found to be inherited, it would be necessary to assume that somatic mutation or some other change has taken place in the genetic constitution during sporophytic development. This has been found to explain the variation of the mosaic pericarp of corn (*2*); and Nagai (*9*) has obtained results which indicate that soy beans may not be entirely free from somatic mutations. However, the writer now has a great many data which show that heritable somatic changes are not the cause of the immense amount of variation in the production of mottling in soy beans.

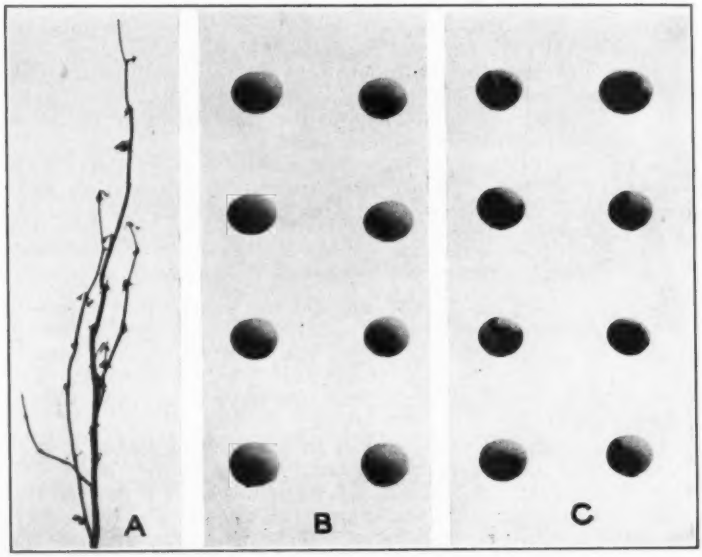

FIG. 1.—A, branch of an Ito San soy-bean plant bearing pure nonmottled seeds; B, typical seeds borne by the branch shown in A; C, typical seeds borne by the other branches of the plant. Plants of this type with branches bearing seeds entirely free from mottling when the rest of the plant bears mottled seeds are not common

In the fall of 1924 the writer became aware of the extreme variability of mottling found in seeds borne by the same plant. In an attempt to determine how general this variability is, several hundred plants were hurriedly examined in the field. In certain progenies, grown on certain types of soil, nearly every bean was mottled and the degree of mottling within the plant was fairly uniform. In other instances it was difficult to find a plant with a uniform lot of seed and occasional plants were found showing remarkable variation.

For a more detailed study, 25 plants showing some variation within the plant were selected, and a careful observation was made of the seed in relation to its position on the plant which bore it. Four plants were found having decidedly more mottled seed at the tip of the central stalk than elsewhere, but other plants showed the opposite tendency and, in general, a random distribution seemed most common.

In the fall of 1925 further studies were made of the distribution of mottled seeds on the plant, and from the evidence obtained it ap-

peared that mottling may in some cases be limited entirely to a single branch. Previously, the plants that were studied were threshed separately by means of a small machine. In 1925, however, the majority of the observations were made on pods picked at random from the plant. This method afforded ample opportunity to detect abnormal conditions, and six Ito Sans and three Mandarins were found which showed mottling only on a certain part. In these cases every seed on the branch was either pure yellow or mottled, and the mottling, where it occurred, was quite uniform (fig. 1).

Distribution of Mottled Seed Within the Pod

In an effort to determine the relation between the occurrence of mottling and the position of the seed in the pod, 14 plants were examined, and each pod was classified. The results obtained are given in Table 1. In this study 2,186 seeds were observed, of which 1,165, or 53.3 ± 0.720 per cent, were mottled. The 1-seeded pods had 56.7 ± 2.18 per cent of mottled seed; the 2-seeded pods, 55.1 ± 1.18 per cent; and the 3-seeded pods, 51.3 ± 1.00 per cent. Although the 1-seeded pods had slightly more mottled seed than the 2 and 3 seeded pods, the difference is not statistically significant. The greatest difference is between the 2 and the 3 seeded pods, which is 3.8 ± 1.55 per cent, and this can hardly be considered significant.

Table 1.—*Relation between occurrences of mottling and position of seed in the pod in seed pods from 6 Mandarin, 5 Ito San, and 3 Manchu plants*

Position of seeds in pods	Number of pods on individual plants of—														Total number of pods	Number of pods calculated on basis of random assortment
	Mandarin						Ito San					Manchu				
1-seeded pods:																
Pure yellow	4	7	4	13	6	10	3	6	0	12	7	12	13	5	102	110
Mottled	4	14	9	14	10	15	2	3	8	12	21	8	4	10	134	121
2-seeded pods:																
Both pure yellow	14	13	7	10	10	10	16	7	2	23	4	12	19	16	163	88
First, pure yellow; second, mottled	0	0	0	1	1	2	2	1	1	0	4	1	1	0	14	101
First, mottled; second, pure yellow	2	3	2	1	2	4	2	0	0	1	2	2	3	0	24	101
Both mottled	5	17	11	13	8	11	1	7	23	9	39	17	13	30	204	115
3-seeded pods:																
All pure yellow	15	5	2	2	2	5	18	56	0	15	0	9	7	13	149	58
First mottled, second and third, pure yellow	4	0	1	1	0	1	7	3	0	0	1	1	0	0	19	66
Second mottled; first and third, pure yellow	1	0	0	1	0	0	2	2	1	0	0	1	0	0	8	66
Third mottled; first and second, pure yellow	0	0	1	0	0	0	1	1	0	2	3	0	0	0	8	66
First and second mottled; third, pure yellow	0	1	1	0	0	1	4	2	3	0	4	1	0	0	17	76
First and third, mottled; second, pure yellow	1	0	0	0	0	1	3	1	0	0	1	1	0	0	8	76
Second and third, mottled; first, pure yellow	0	0	3	2	0	0	1	2	0	0	4	0	1	0	13	76
All mottled	3	11	5	2	3	4	6	7	56	4	19	12	8	18	158	86

Although the number of seeds in a pod does not seem to be a significant factor, it will be noted in Table 1 that in both the 2 and 3 seeded pods there is a strong tendency for the seeds in any one pod to be either all mottled or all nonmottled. If the probability of a seed being mottled is 0.533 and the probability of being nonmottled is 0.467, the probability of any combination can be readily calculated if mottling occurs at random. To illustrate the principle: A 3-seeded pod with the first seed mottled, the second nonmottled, and the third mottled, would have the probability of occurrence of $0.533 \times 0.467 \times 0.533$, that is, 0.133. The calculated results, assuming random assortment, are given in Table 1 for comparison with the observed.

The results given in Table 1 show that there is no particular position in the pod that is more likely to produce a mottled seed than any other position. In contrast with these results, Halsted (*3*) found that the position in the pod is important in determining the size of a seed. In both 2-seeded and 3-seeded pods he found that the basal seed was most likely to be the smallest and the seed at the tip of the pod the largest. In order to account for all of these results it is necessary to assume that mottling is not necessarily associated with the size of the seed.

Table 1 does not show the differences in degree of mottling, since every seed was classified as either pure yellow or mottled. Woodworth and Cole (*18*) go a step further and show that the type and degree of mottling within the pod is frequently uniform. The general conclusion can therefore be drawn that every pod is subjected to physiological conditions which affect the seeds within that pod in a similar manner. In plants that bear both mottled and nonmottled seed a very delicate balance must exist between the physiological conditions that cause mottling and those that prevent it. All the seeds on any one plant would surely be supplied with similar nutrients, and it is only when a very slight difference is significant that such striking contrasts should be produced as those exhibited by certain soy-bean plants.

ABNORMAL PHYSIOLOGICAL CONDITIONS

Mention has just been made of certain cases in which the mottled seeds were localized on certain branches, but in these cases the mottling was no more severe than is commonly found. On certain abnormal plants, however, the mottling was much worse than that which ordinarily occurs, showing the extreme effect which may be produced by environmental conditions. It is difficult to determine all the causes of this abnormal production of pigment, but the accumulation of synthetic products, especially sugars, seems the most probable underlying factor.

Plants with abnormal foliage have not been uncommon. Many developed crinkled leaves late in the season, but some exhibited an abnormal appearance from the seedling stage. In a few extreme cases the leaves were badly curled and spotted, presenting an appearance much like that of tobacco and potato plants infested with a mosaic virus. No success was attained, however, in transmitting the malady from one plant to another.

In the fall of 1924 one of these abnormal plants was found in a progeny of Manchu. This progeny had been selected for a uniform yellow seed coat and black hilum. It has since proved to be free from bad mottling, and subsequent progenies have bred true to type. The abnormal plant was very much stunted and none of the pods were normally filled out. It was about November 1 when the progeny was harvested, and at this time all the plants were perfectly mature except this abnormal individual. This plant alone was green, and although it had a sickly appearance it was undoubtedly manufacturing food material. The pods, however, had already turned yellow and had come to maturity. When these were opened the seed was found to be almost entirely self-black, exhibiting a very striking contrast to the pure yellow seed of sib plants. The most natural assumption was that this plant must have been a rogue. The black seed, therefore, was planted in 1925. The resulting plants produced seeds as free from mottling as those of the grandparent, showing conclusively that the plant with the black seeds was not a rogue and that the extreme type of mottling was due to physiological conditions rather than to heredity.

Other plants which appeared to be seriously diseased were found in strains of Ito San and Mandarin in 1924, but the degree of mottling did not seem to be markedly affected by the condition of the plant. In 1925, however, two Mandarin plants were found which had very much the same diseased appearance and their seeds were nearly self-brown.

In the fall of 1925 abnormal conditions were again observed, and these throw considerable light on the previous results and prove beyond all doubt that physiological factors may greatly influence pigment formation. Three Manchu plants were found on which some of the branches were broken over, and striking illustrations of variation of mottling within the same plant were obtained. The seeds on the broken branches were entirely normal, while those on the remaining central part of the plant were small and almost entirely black, presenting the same appearance as the seed of the abnormal Manchu plant found in 1924 which has just been described.

Figure 2 shows one of the abnormal plants as it grew in the field, and the contrast in the appearance of the seeds caused by the abnormal physiological conditions. That the branches had not recently been broken was evident from the nature of the breaks, yet all the pods on the broken branches were well filled out. The central part of each of the three plants appeared somewhat diseased; about half the pods failed to develop, and none were normally filled out. The leaves on the central stalk continued to grow after those on the broken branches had matured. It is very unlikely that a disease organism or virus was involved in bringing about the abnormal condition in the central stalks, for if such had been the case all the vascular tissue of the plant would have been affected.

Since the abnormal portion of these plants was green and was still manufacturing plant food, one would expect the amount of sugars as well as of other food materials to be increased. That the seeds were mature has already been mentioned, and the nature of the growth resembled very much that of sterile plants where no flowers are set and nothing but an accumulation of food material in the stems and leaves is possible.

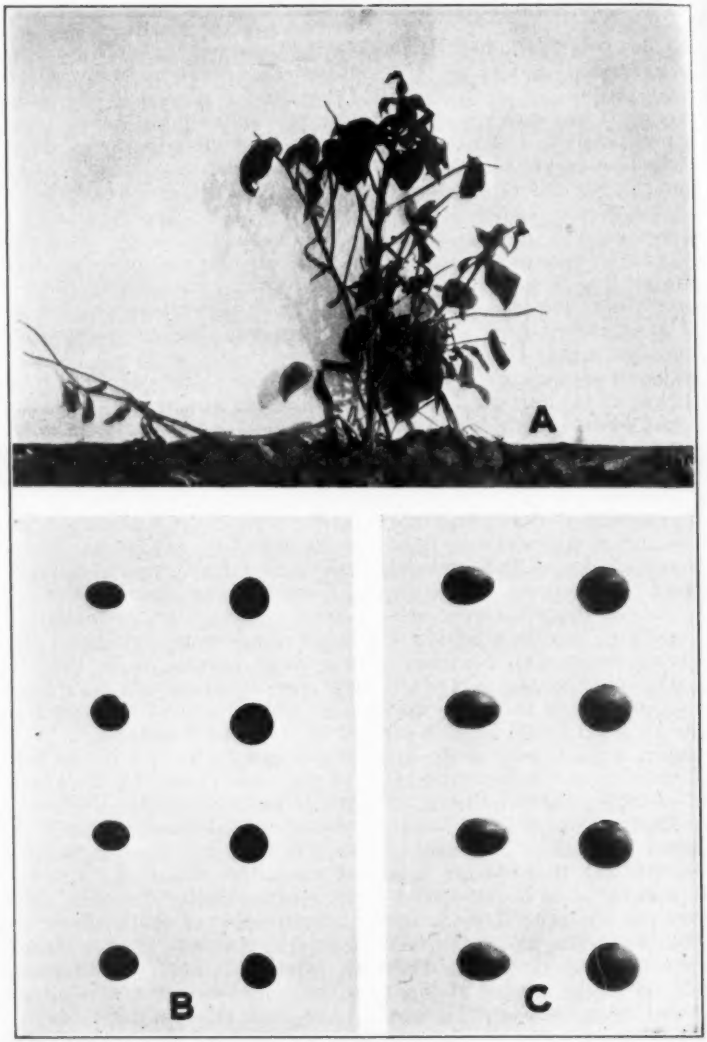

FIG. 2.—A, one of three abnormal Manchu plants of the same type found in 1925. The branches that were broken over produced normal seed while the remaining portion of the plant bore seed that was almost entirely black. B, typical seed from the central part of the plant. C, typical seed from the branch which was broken over

The reason why the pods matured before the leaves is not entirely clear. It is possible that the abnormal condition may have caused a deficiency of moisture and plant-food material earlier in the season and when the plant recovered and again resumed vigorous growth, the seed was physiologically mature and unable to make further growth. Moisture and food material supplied to these mature embryos at this time could result only in germination, as has been observed in the greenhouse when water is applied to mature plants. The nature of the enzymatic changes that take place in maturation make further growth impossible. The seed coat, however, is a tissue that easily absorbs moisture, so it seems entirely possible that an abnormal condition of this kind might markedly increase the concentration of sugars. If such an assumption is correct the excessive development of pigment in the seed coat might logically be expected.

Attempts have been made to produce abnormalities by thinning the pods on a plant in the expectation that the addition of food material which each seed would get would cause an increase in the quantity of pigment formed. Two Mandarin plants were treated in this way and expectations were fully realized. Figure 3 shows how the seeds from one of these plants increased in size and became nearly self-brown. On the other hand, many plants were treated in the same way and no effect on the amount of mottling was observed. There is room for a great deal more work along this line, but the results so far, although very poorly understood, are unique in demonstrating that mottling is not necessarily determined by the amount of food material present. Many very large seeds were produced by plants the pods of which were thinned without any apparent increase in the amount of pigment formation. The chemical balance of this food material therefore must also be important.

EFFECT OF LARGE AND SMALL QUANTITIES OF NITROGEN

Since a high relative amount of carbohydrates in comparison with nitrogen is known to affect the production of pigment in the leaves and stems of many plants [8] (*11*), it seems that the production of pigments in the seed coat should be similarly affected. If soy-bean plants could be grown with a high relative amount of nitrogen there should be little chance for carbohydrates to accumulate, with a possible corresponding absence of pigmentation.

With these facts in mind a project was undertaken in 1924 to determine, if possible, the effect of the carbohydrate-nitrogen relation on mottling. Two-gallon glazed pots filled with pure quartz sand were employed for this purpose, and the chemicals were applied according to the appearance of the plants. In this way plants were grown which were nearly yellow for lack of nitrogen and others were made very succulent by adding liberal quantities of nitrogen.

After ripening, the seeds from each plant were examined, but the data obtained were very conflicting. Some of the plants given only a very small amount of nitrogen produced mottled seed, and some given a large amount produced mottled seed. Plants bearing seed free from mottling were also produced under both conditions. The results indicate that the carbohydrate-nitrogen relation was probably not a factor in determining mottling, but the nature of the experiment

[8] MacGillivray, J. H. EFFECT OF PHOSPHORUS ON THE NUTRITION OF THE TOMATO PLANT. 1925. [Unpublished doctor's thesis. Copy on file, library, Univ. Wisconsin, Madison.]

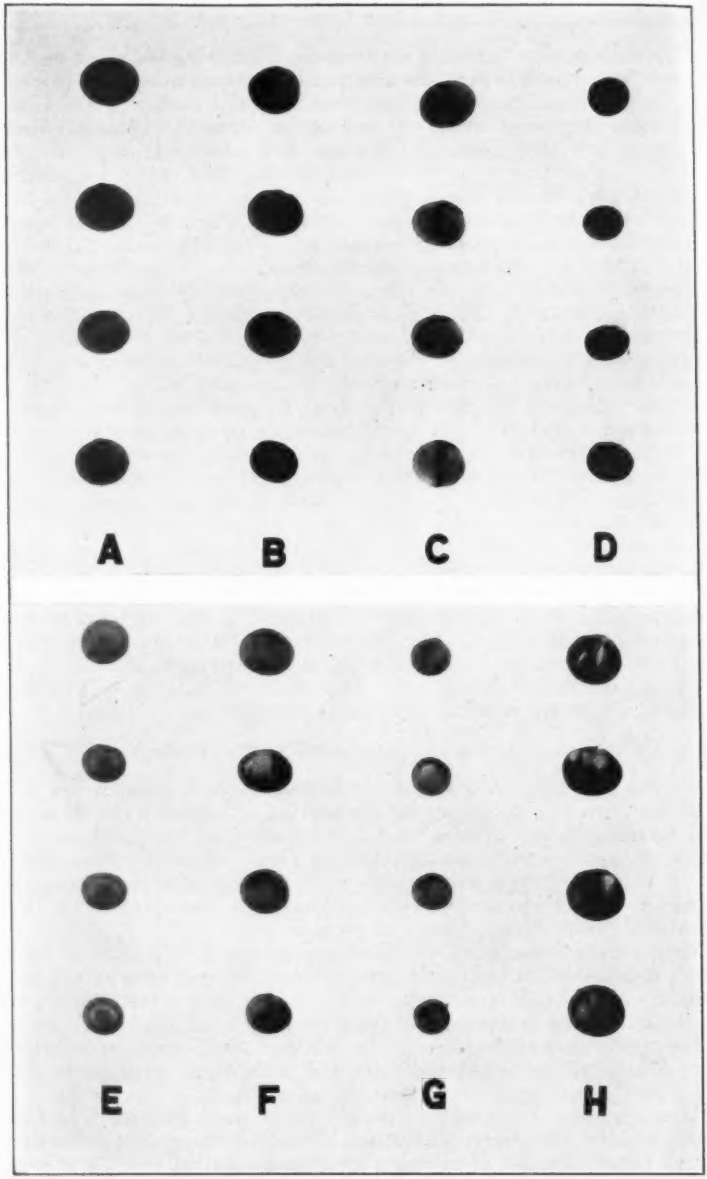

FIG. 3.—A to D, variation in mottling occurring in No. 2 strain of Manchu soy bean: A, seed from plant grown on sandy soil; B and C, seed from plant grown on rich soil; D, seed from an abnormal plant that presented a diseased appearance but that continued growth after the seed was mature. E to H, variation in mottling occuring in Mandarin variety: E, seed from plants grown close together in the row on a sandy type of soil at Spooner, Wis.; F, seed from plants grown on same type of soil as E, but spaced 3 feet apart in the row; G, small seed produced on uninoculated soil by plants that did not produce a thrifty growth; H, large badly mottled seed produced by thinning pods on a plant

does not warrant a definite conclusion on this point. It was very difficult to keep the plants growing uniformly, even those in the same pot, and this was particularly true as the time of maturity approached. All the nitrogen-high Mandarins, however, matured normally and very little mottling was noted on the seeds from these plants, but Ito San and Manchu plants grown under the same conditions were very much delayed in maturing and considerable mottled seed was produced. The time and nature of maturity, therefore, was probably a factor in the production of mottling, and it seems likely that it might have been more important than the condition of the plant in its earlier development.

TYPE OF SOIL AND DISTANCE BETWEEN PLANTS

In 1923 progeny tests were being run with Ito San and Mandarin seeds in cooperation with the United States Department of Agriculture. Three kinds of Ito San seed were planted, one of which had been breeding true for pure yellow seed. The other two lots had shown mottling, but in one case the mottled seeds were selected, and in the other case the nonmottled. Table 2 shows the behavior of these three selections when grown at Madison, Wis.

TABLE 2.—*Number of plants producing pure yellow and mottled seed in progenies from pure-line yellow, yellow, and mottled seed of Ito San soy bean, grown at Madison, Wis., in 1923*

Plot where grown	Seed planted	Number of plants producing pure yellow seed	Number of plants producing mottled seed	Per cent of plants producing mottled seed	Statistical comparison with pure-line yellow on Plot A			
					Difference in per cent	Probable error of difference in per cent	Difference ÷ probable error	Odds (approximate)
Plot A	Pure-line yellow	5	22	81.5				
	Yellow	6	23	79.3	−2.2	7.4	0.3	
	Mottled	2	20	90.9	+9.4	6.6	1.4	2:1
Plot B	Pure-line yellow	45	18	28.6	−52.9	7.4	7.1	435,000:1

On the same type of soil there was no significant advantage noted in the specially selected strain. A planting of the pure-yellow selection happened to be repeated on another plot, however, and an increase in mottling amounting to 52.9 per cent was obtained. This is over seven times the probable error of the difference to be expected from chance, and is surely significant.

Not only was there more mottling on one plot than on the other, but the degree of mottling was worse in plot A than in plot B (Table 2). It is not clear just what the difference in soil type was. A very thrifty growth was produced in both instances, and if anything, the plot producing the least mottling was the richer; but definite information is not available. The only point to be made is that mottling was affected by soil conditions.

During the summer of 1924 a study was made of the effect of soil-nutritional conditions. Five strains of seed were used in this project. Four single plants were chosen for four of the strains and a mixed sample was used for the fifth. One strain was selected from the pure-line Mandarins and another from the pure-line Ito Sans that

had been obtained from W. J. Morse, of the United States Department of Agriculture. Two types of Manchu were grown, No. 1 and No. 2. Strain No. 2 produced a larger plant and required a growing period approximately two weeks longer than strain No. 1. The mixed sample was from a pure line of Mandarins obtained from E. J. Delwiche, of the Spooner, Wis., station. No mottling had previously been observed in this strain at the Spooner station, where the selection was made.

One plot was selected on a sand hill where there was very little chance for vigorous growth. The plants managed to survive and grew to a height of about 10 to 12 inches, but very few seeds were set. A second plot was selected on a rather heavy clay soil. Here the plants grew to a height of 16 to 22 inches. The Mandarins seemed to be particularly affected by this type of soil, for they were very much smaller than the Ito Sans and Manchus, which was not the case on the rich soil. For the third plot a rich loam soil that had been heavily fertilized was chosen. Here all the plants grew vigorously and the second strain of Manchus reached a height of 28 inches. On the second and third plots the plants were all spaced 3 feet apart, but on the sandy soil they were planted only 1 foot apart because of lack of space.

TABLE 3.—*Effect of soil type and thickness of stand on mottling in soy-bean seed*

[Summary of results obtained in 1924 with Mandarin, Ito San, Manchu No. 1, and Manchu No. 2]

Type of soil and distance apart of plants in row	Height of plants in inches (approximate)	Total plants threshed	Per cent mottled [1]	Per cent medium mottled or worse
Light sandy soil; plants 1 foot apart	10–12	66	6	1
Heavy, rather infertile clay soil; plants 3 feet apart	16–22	226	66	31
Rich loam soil; plants 3 feet apart	24–28	125	100	90
Rich loam soil; plants approximately 1 inch apart	40–50	161	26	12

[1] Individual plants were arbitrarily classified as nonmottled, slightly mottled, medium mottled, and badly mottled; so the percentages refer to the number of plants rather than to the total number of seeds.

It will be noted in Table 3 that practically all the plants on the sandy soil produced pure yellow seed. Mottling occurred on the plants grown on the heavy clay soil, but most of it was rather faint, especially in the case of the Mandarins. On the rich soil, however, every plant produced mottled seed, and in 90 per cent of these cases the mottling was bad.

At the time that these observations were made varietal tests were in progress with certain Ito San, Mandarin, and Manchu selections growing on rich soil not far from the plot where mottling occurred in such abundance. These varieties were grown from seed very closely related to that which was used in the mottling project; so a comparison is not out of place. In the varietal tests the plants were close together in rows 3 feet apart and the rate of seeding was 1 bushel per acre. A very different type of plant from one grown 3 feet apart was produced under such conditions. The stems were very much thinner and the height of the plants was 40 to 50 inches, or nearly twice that of the plants spaced 3 feet apart. The growth was in many ways

similar to that obtained by growing plants in the greenhouse under reduced light. The number of seeds per plant was greatly decreased and the seeds themselves were much smaller.

Table 3 gives the combined results for the Ito San, Mandarin, and Manchu varieties spaced 1 inch apart. Eighty-eight per cent of these plants bore seed practically free from mottling, whereas a very similar soil failed to produce a single plant that did not show mottling when spaced 3 feet apart. The matter of thickness of stand, as well as the type of soil, therefore, appears to be an important factor in the production of mottling.

In 1925 an attempt was made to repeat the work done in 1924, and to get additional information in regard to the effect on mottling in seeds from plants grown on different types of soil with different distances between them. Table 4 gives some of the results. The original strains of Ito San and Manchu were used in this project, and a selection of Mandarins was made from the pure line obtained from the Spooner station. In addition to observations on selected plots of different types of soil at Madison, a test was run at the Spooner station, where mottling had never previously been observed to any great extent.

TABLE 4.—*Comparison of amount of mottling produced on seeds of different varieties of soy beans when plants are spaced at different distances on various types of soil; 1925 experiments*

Description of soil	Distance apart in row	Variety	Number of plants bearing seeds which showed—				Total plants observed	Per cent mottled
			No mottling (pure yellow)	Slight mottling	Medium mottling	Bad mottling		
Rich loam, Madison, Wis. (inoculated).	3 feet	Mandarin	3	9	12	0	24	88
		Ito San	0	0	0	25	25	100
		Manchu No. 1	10	4	5	0	19	47
		Manchu No. 2	3	10	3	1	17	82
	4 inches	Mandarin	3	6	2	0	11	73
		Ito San	0	0	0	25	25	100
		Manchu No. 1	19	0	0	0	19	0
		Manchu No. 2	2	6	3	0	11	82
Rich loam, virgin to soybeans, Madison, Wis. (not inoculated).	3 feet	Mandarin	5	8	5	0	18	72
		Ito San	0	0	0	50	50	100
		Manchu No. 1	30	0	0	0	30	0
		Manchu No. 2	2	11	30	5	48	96
	1 inch	Mandarin	10	19	17	0	46	78
		Ito San	5	5	26	4	40	88
		Manchu No. 1	25	0	0	0	25	100
		Manchu No. 2	4	7	23	4	38	89
Light and sandy, Madison, Wis. (inoculated).	3 feet	Ito San	11	0	6	0	17	35
		Manchu No. 1	33	0	0	0	33	0
		Manchu No. 2	12	1	3	0	16	25
	1 inch	Ito San	0	6	20	0	26	100
		Manchu No. 1	57	6	0	0	63	10
		Manchu No. 2	0	7	18	0	25	100
Light and sandy but in high state of fertility, Spooner, Wis. (inoculated).	3 feet	Mandarin	6	9	5	0	20	70
		Ito San	0	0	0	25	25	100
		Manchu No. 1	23	0	0	0	23	0
	1 inch	Mandarin	100	0	0	0	100	0
		Ito San	53	0	0	0	53	0
		Manchu No. 1	25	0	0	0	25	0

From Table 4 it is apparent that the plants on sandy soil again showed a tendency to produce seed free from mottling, regardless of whether they were spaced 1 inch or 3 feet apart. From August 8 to September 6 there was only 0.16 inch of rainfall at Madison, and

the effect on the soy-bean plants grown on the sandy soil was noticeable. After September 6 when rains again fell certain plants began to take on a vigorous growth. This growth affected the plants in such a way that there were marked differences in the time of maturity. The plants spaced 3 feet apart set many more seeds, survived the drought in a more normal condition, and matured earlier than those that were spaced only an inch apart. This was particularly apparent on the sandy soil in the case of Ito San, Mandarin, and Manchu No. 2.

The first strain of Manchus, as already mentioned, matures earlier than the second. This was especially noticeable on the sandy soil at Madison where the first strain was too nearly mature to be revived by the September rains, and the seed was entirely free from mottling. The Mandarins were more affected by the drought than the other varieties. In most cases less than six seeds were set per plant and some plants failed to set any, but after the heavy rains in September all these plants took on a dark green color and began to grow. The seeds produced, while few in number, were even larger than normal seed and were badly mottled. The Mandarins on the sandy plot produced so small a quantity of seed that the data for this variety are not given in Table 4. Very similar results, however, were obtained in the case of Ito San and the No. 2 strain of Manchu.

Unless all the conditions of the experiment are considered, a hazy idea is likely to be obtained from Table 4 as to the effect on the plants of the difference in distance between them. It is evident that spacing is a factor of importance only in certain cases. On the rich soil all four strains mottled so badly that no difference resulting from spacing was noticeable except in the Manchu No. 1 strain. Here the seeds from 47 per cent of the plants 3 feet apart produced mottling, while all seeds from those grown 1 inch apart were pure yellow. The opposite condition was observed on the sandy soil, as already explained, plants spaced 3 feet apart producing the purer seed.

The most striking effect of spacing was noted at the Spooner station. Seeds of the first strain of Manchu were pure yellow even when planted 3 feet apart; but all the Ito Sans and Mandarins grown 3 feet apart tended to be badly mottled; while the Ito Sans and Mandarins closely spaced, in rows grown for varietal trials, produced seed entirely free from mottling. It should be mentioned that the type of soil at the Spooner station was a light sandy loam but well fertilized. Since mottling appeared in a marked degree on the Ito San and Mandarin varieties when spaced 3 feet apart, it may be assumed that the reason why mottling had never been observed there before was because all observations had been made on plants grown close together for varietal trials.

Table 4 also shows the results obtained from growing the same varieties on a soil virgin to soy beans without inoculation, all other plots being inoculated. The plants on this plot were not particularly thrifty, yet mottling was very prevalent in all varieties except the No. 1 strain of Manchu. Plants grown on this plot were so poorly nourished that the seed was often very small, but at the same time it was badly mottled. In previous instances mottling seemed more likely to be associated with large seed produced on rich soil. This point is of particular interest because it shows again that the total

amount of nutrient material may not be the determining factor in mottling. The seeds produced by the same strain on the sandy soil were well filled out in many cases and at the same time were free from mottling.

TABLE 5.—*Effect of thickness of stand on mottling of seed in a commercial variety of Manchu soy bean*

Distance apart of plants in row	Number of plants producing seed that showed—				Total	Per cent mottled
	No mottling (pure yellow)	Slight mottling	Medium mottling	Bad mottling		
3 feet	34	14	18	1	67	49
1 inch	160	3	0	0	163	1.8

Table 5 shows better than Table 4 the effect on mottling of the distance between plants in the row. In the experiment reported in this table an ordinary variety of Manchu was used in which there may have been unrelated strains, but the numbers are large enough to give a good random sample in each case. Two rows of the ordinary thick planting used in varietal trials were simply thinned so the plants were 3 feet apart in the row. The table shows that seed from plants grown close together was much freer from mottling than seed from plants grown farther apart.

FERTILIZERS

Additional work was carried on in 1925 to determine the effect which fertilizers might have on mottling. On the rich soil at Madison a row of each of the four strains used in the other trials was treated with a heavy application of sodium nitrate. A test was also run on peat soil with applications of phosphorus and potassium to determine the effect of these fertilizers on a soil in which they are supposedly lacking. Most of the plants produced on these soils bore mottled seed, but there was no apparent effect from the fertilizers in any case. The fact that the results were negative does not prove, however, that these fertilizers may not be important factors in producing mottling; but simply that they appeared to exert no influence in these particular cases.

INOCULATION

Insufficient work has been done to allow definite conclusions to be drawn as to the effect of inoculation on mottling, but there is good reason to believe that it is an important factor. On rich soils where mottling has been pronounced, nodule production has been poor, and on sandy soils where little mottling has been observed nodule production has been exceptionally good.

No satisfactory explanation has been given as to why there is poor nodule production on certain types of soil, yet considerable difficulty has been experienced in growing soy beans because of this fact. Perhaps the most illuminating work in this field is that concerned with the behavior of different strains of nodule-producing bacteria. Wright (*19*, *20*) has studied and described two types of *Pseudomonas*

radicicola, the bacterial organism causing nitrogen-fixation in soy beans. Three years' work showed that plants inoculated with type A fixed much more nitrogen than plants inoculated with type B.

A possible explanation may be given, therefore, of a very common observation, that is, in cases where no mottling occurs the first year soy beans are grown, in later years it may become very pronounced. If a poor nitrogen-fixing strain of bacteria should gain preponderance over the original good nitrogen-fixing strains, one would naturally expect mottling to be affected.

SHADING

In 1923 approximately 300 plants, including all the strains that were being tested for the inheritance of mottling, were bagged to insure self-pollination. After the pods had set and the bags were removed, it was apparent that the shading had caused the stems to be finer in texture, but that of all the 300 plants only 4 were free from mottling. Very similar results were obtained in 1924, about the same number of plants having been bagged. Hollowell's[9] observations in regard to shading accord with these results.

Although bagging in the field did not prevent mottling, very different results were obtained by growing plants in the greenhouse. In the winter of 1923–24 a number of varieties, together with some hybrids, were grown in the greenhouse with the aid of artificial light. A genetic study was the chief object of these experiments, but the observations on mottling are of interest as well. Approximately 250 plants were grown and not the slightest sign of mottling was noted in any of the seeds produced. The same number of plants were grown in 1924–25 with almost the same results. It is difficult to explain these findings, but since the plants produced a much more spindling growth one would expect that light intensity was an important factor.

To get more information regarding light intensity, six plants from each of the four strains used in the mottling test in 1925 were grown under a frame covered with three thicknesses of cheesecloth. The plants grew normally under these conditions, the only noticeable differences being a tendency to grow higher and to produce slightly weaker stems. All of these plants produced mottled seed, and although the amount of mottling may have been slightly less than in the controls, the difference was not significant.

Although it seems logical that shading should be an influencing factor under certain conditions, all attempts to reduce the amount of mottling by shading in the field have failed. Of course, the length of the light wave as well as the intensity of the light may influence mottling, but this factor has not been taken into account. Since a large percentage of the ultra-violet light is screened out by ordinary glass it may be possible that mottling did not occur in the greenhouse for this reason; but studies under controlled conditions are necessary before any proof can be established.

[9] HOLLOWELL, E. A., FACTORS INFLUENCING THE MOTTLING OF THE SOY BEAN SEED COAT. 1924. [Unpublished master's thesis. Copy on file, library, Iowa State College, Ames.]

HEREDITY IN RELATION TO MOTTLING

Attention has been called to the wide variation in mottling, which may be caused by environmental conditions. It has been proved that these variations may occur in the same pure-line selections independent of hereditary influences, but the differences which have been observed among different varieties and among selections of the same variety show that there is also a tendency for mottling to be inherited.

During the course of this study considerable effort has been made to select for and against mottling. These efforts have met with remarkable success, but not within related progenies. When once a selection was made the tendency was to breed true except for the variation that could be accounted for as due to environmental influences. One selection alone (No. 83 in the Black Eyebrow variety) showed signs of segregation. The evidence indicates that the original seed selected in this case was heterozygous, but all the other numerous selections were apparently homozygous to start with. For this reason mottling can not be attributed to hybridization or to mutations, at least not the large amount that is commonly observed. This does not mean, however, that mutations or hybridization may not occasionally occur, but it is unlikely that they occur with very great frequency.

Genetic analyses (*13*) revealed the important fact that the self-color type in soy beans is a recessive character and that a multiple series of factors is responsible for preventing the development of pigment in the seed coat. I^h is the factor which tends to inhibit all pigment fomation, I^1 inhibits pigments to the hilum, I^k inhibits them in such a way as to form an "eyebrow" pattern, as in the Black Eyebrow variety (fig. 4), and i is the recessive factor which has no effect. With an allelomorphic series of this sort all gradations of restriction factors may be imagined, and this seems to be the logical interpretation of the results which have been obtained. Mutations within such an allelomorphic series would not be unexpected and this would provide a method by which heritable differences may occasionally be produced.

Although these restriction factors (I^h, I^1, and I^k) have been spoken of as dominant, it should not be implied that they are fully dominant. The relationship of I^k to i has not been established, but I^h and I^1 are only partially dominant over i. Therefore, hybridization with a self-colored variety increases the mottling in the F_1 plant (fig. 4).

Besides this allelomorphic series which inhibits the development of pigment, there are other factors that may be considered as modifying its development. The most important of those which have been identified is the factor T, for tawny pubescence. This factor affects both the quality and quantity of pigment formed. It is possible that there are other factors in the same class and the relationship may be very indirect. Since mottling is known to be easily influenced by any change in the nutritional balance of the plant, there is considerable opportunity for factors primarily concerned with the physiological behavior of the plant to have a modifying effect on mottling.

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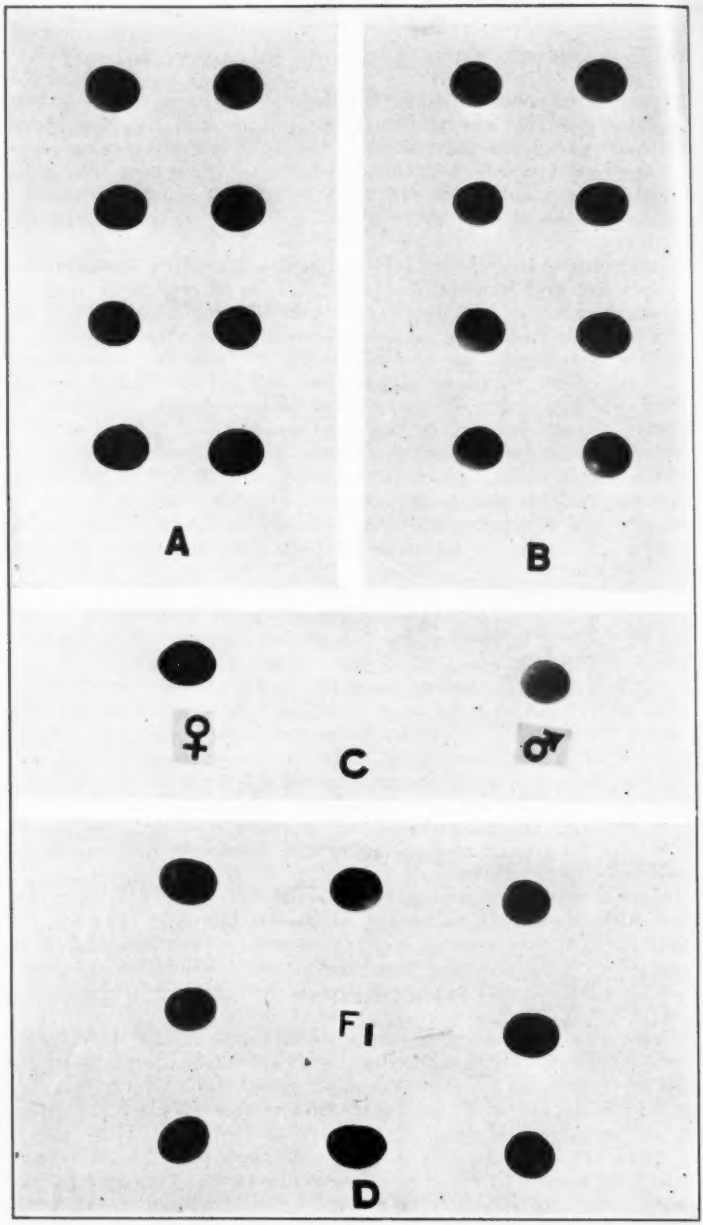


FIG. 4.—Differences found to be due to heredity in the "eyed" or "patched" pattern of the Black Eyebrow variety of soy bean: A, nearly black seeds typical of strain No. 82; B, seeds with typical uniform pattern from strain No. 84. C, ♀, a black seed from a selection of an unknown variety; ♂, a Mandarin; D, F_1 seeds, showing some of the variations in mottling found in the hybrid plant

From the limited amount of work so far accomplished it is difficult to distinguish the influence of heredity from that of environment. Environmental influences are so strong that it is impossible to form any judgment of the hereditary potentialities by the appearance of the seed from any one plant. The only safe method is to grow a number of seeds from the plant in question and to make statistical comparisons with other progenies for the amount of mottling produced. This is necessarily slow work and the number of progenies that can be tested is limited.

SELECTING AGAINST MOTTLING

In 1922, 206 plants were grown from seed selected for different types of mottling, and 94 of these were bagged with cheesecloth to prevent cross-pollination. Most of the plants grown from this seed were badly mottled and only 10 were free from mottling. The type of mottling varied greatly, only 4 plants breeding true for the particular pattern of the parental plant.

In 1923 a further attempt was made to study the inheritance of mottling in these selections. As a general rule the mottling had been so pronounced that an effort was made to select out nonmottled strains. In 1922 certain progenies of Manchu had seemed to be much more susceptible to mottling than others. From the seed produced eight progenies were grown for comparison with other selections which showed a tendency to be free from mottling. Table 6 gives the results of these experiments. Considerable variation was noted within each of these progenies, but Table 6 shows that there was much more mottling in the eight selections than in the other progenies. This means that there must have been heritable differences, but each selection seemed to breed approximately true to the original type. Since there were no signs of segregation, it is logical, in spite of evidence of heritable differences, to assume that the original selections were homozygous and that the variations which occurred within each selection were due to environment.

TABLE 6.—*Degree of mottling apparent in progenies from unmottled seed of low-mottled strains, mottled seed of low-mottled strains, and mottled seed of high-mottled strains of Manchu soy beans; 1923 experiments*

Parental stock	Number of progenies grown	Number of plants with seeds that showed not more than a trace of mottling	Number of plants with seeds that showed slight mottling	Number of plants with seeds that showed medium or bad mottling
Nonmottled seed from low-mottled strains	23	153	54	32
Mottled seed from low-mottled strains	15	63	26	10
Mottled seed from high-mottled strains [1]	8	0	7	120

[1] The plants from the high-mottled strains which were selected for this study were comparable to the more badly mottled plants from the low-mottled strains.

In 1924 two strains of Manchu were selected for study, No. 1 and No. 2. Strain 1 was originally nearly free from mottling and throughout all the tests that were made in 1924 and 1925 it continued to breed true, except under environmental conditions most favorable

for mottling. Strain 2, however, which was originally badly mottled, constantly produced bad mottling except under environmental conditions unfavorable for the development of mottling.

Selection within varieties other than Manchu has been attempted, but no success has been attained. In 1923, 43 progenies were grown from plants which had produced brown mottling. Ten of these were probably Ito Sans and one was evidently a selection from the Aksarben variety; the other 32 progenies were not identified as belonging to any particular variety.

Only three plants from the Aksarben strain matured in 1923. These plants produced pure yellow seed, but in 1924 and 1925 all Aksarben progenies were mottled. The Ito San progenies in 1923 also showed some freedom from mottling and two plants bore pure yellow seed. From Table 7 it is apparent that environmental influences must have been responsible for the fact that these plants produced pure yellow seed, because in 1924 their offspring were just as badly mottled as the progenies from plants showing bad mottling in 1923. The 32 progenies grown in 1923 which could not be identified with any particular variety, continued to produce mottling in 1924. There was often considerable variation from one plant to another in the same progeny, but this was to be expected since environmental conditions were not entirely uniform.

TABLE 7.—*Number of plants producing mottled seed in progenies grown from (1) medium to badly mottled seed derived from strains showing some freedom from mottling, (2) medium to badly mottled seed derived from strains showing no freedom from mottling, and (3) seed free from mottling; Ito San variety of soy bean*

Parental stock	Number of progenies grown	Number of plants producing seed that showed medium or bad mottling	Per cent of plants producing mottled seed
Medium to badly mottled seed, from strains showing some freedom from mottling	4	98	100
Medium to badly mottled seed, from strains showing no freedom from mottling	4	56	100
Yellow seed free from mottling	2	33	100

These results show that it has been possible to select for strains that are more inclined to mottle than others. The seed from which selections were made, however, may have been unrelated, so it can not be said that there has been successful selection within any one related line. The original seed in these cases must have been homozygous. If heterozygous material had been selected instead of homozygous it is entirely possible that selections could have been made within individual progenies. If mottling were produced by frequent somatic mutations this should also have made it possible to select for or against mottling.

SELECTING FOR PIGMENTATION PATTERNS

A random assortment of seed from the Black Eyebrow variety was planted in 1922 and six plants, Nos. 81 to 86, were grown to maturity and threshed. These plants were spaced 3 feet apart and were

bagged with cheese cloth during the flowering period. After threshing, considerable variation was observed among the six plants, but No. 84 was different from the rest in that its mottling pattern was reasonably uniform and somewhat lighter.

A progeny from each of the first four plants was grown in the same manner in 1923 except that it was impossible to bag every plant. After threshing and classifying according to the uniformity of the pigment pattern, 17 plants grown from No. 84 were found to have regular and uniform patterns like that of the parent. In contrast with this light uniform pattern, 17 plants from No. 82 produced seed of which much was entirely black.

Nine plants from No. 81 and nine from No. 83 showed considerable variability. Eleven of these plants showed a reasonably uniform pattern while the remaining seven produced very dark-colored seed. Figure 4, A and B, illustrates the difference in appearance bet een the uniform pattern of No. 84 and the dark pattern of No. 82.

TABLE 8.—*Number of plants in progenies of Black Eyebrow soy beans producing seeds with typical eyebrow pattern, pattern slightly extended, mostly obliterated, and quite obliterated*

Parental stock	Number of progenies grown	Number of plants producing seed of—			
		Typical eyebrow pattern [1]	Pattern slightly extended	Pattern mostly obliterated	Pattern quite obliterated (seed nearly black)
Plants with uniform patterns, from No. 84	5	271	7	0	0
Plants with uniform patterns, from No. 83	9	212	138	13	0
Plants with dark seed, from No. 83	4	0	6	122	12
Plants with variations in patterns, from No. 82	2	0	0	51	2
Plants with very dark seed, from No. 82	4	0	0	105	61

[1] See Figure 4.

The next season (1924) 2 progenies from No. 82, 8 from No. 83, and 2 from No. 84 were tested. Table 8 gives the results of these experiments as well as the data from further work in 1925. That these three strains of Black Eyebrow had different hereditary potentialities for the development of a mottling pattern is evident from the table. All of the 278 plants from No. 84 showed a high degree of uniformity, but those from No. 82 continued to produce dark seed, and not a single plant of the 219 grown from No. 82 was comparable in pattern type with the No. 84 strain.

The behavior of progenies from No. 83 indicates very strongly that the original seed selected was heterozygous, but it is the only example of successful selection toward a particular mottling pattern that the writer has observed. None of the progenies from No. 83 produced seeds quite as uniform as those from No. 84, but some were practically as dark as those from No. 82. Three progenies from No. 83, selected for a uniform pattern, continued to produce a uniform pattern in 1925 and two progenies from the dark-seeded plants continued to produce dark seeds.

Although it seems logical to assume that No. 83 was originally heterozygous, it would be useless to theorize on the factorial relationships because of the nature of the variability and the limited number of progenies grown. Heredity is undoubtedly important in producing different amounts of pigment but all other selections happened to be made from homozygous material that was no longer amenable to selection. This example alone affords proof of successful selection within a related strain, and the fact that No. 82 and No. 84 remained constantly different is also proof of hereditary differences.

RELATION BETWEEN COLOR OF PUBESCENCE AND MOTTLING

Among the varieties subject to brown mottling there are noticeable differences in shades and intensities. The stage of maturity and abnormal weather conditions at harvest time may influence the color, but there are very constant differences between certain varieties. Nagai (*8*) has called attention to some of these differences and has determined the genetic behavior of certain browns, buffs, and reddish browns.

In the determination of the shade of brown pigment, the color of pubescence is important. Mandarins, Aksarbens, and certain Chinese varieties that have been grown have a gray pubescence and produce a very light-brown mottling. This light-brown color is the "ochraceous tawny" of Ridgway (*15*). Ito Sans, however, have a tawny pubescence, and the mottling color "auburn" is much more intense than that produced by gray-pubescent varieties.

Not only is the mottling darker on varieties with tawny pubescence but the tendency to produce mottling seems to be more pronounced, at least on certain types of soil. Experiments have shown that Ito Sans are usually more likely to produce mottled seeds than Mandarins. The gray-pubescent varieties other than Mandarin have not been so extensively studied, but they also seem to be freer from mottling than varieties with tawny pubescence.

A hybrid between an Ito San and Aksarben is of interest in this connection. The F_1 plant had tawny pubescence like the Ito San parent, but it was grown in the greenhouse where only a trace of mottling developed. In the F_2 population 59 plants were grown and a classification based on color of pubescence and degree of mottling gave the following results:

	No mottling	Slight mottling	Medium mottling	Bad mottling
Tawny-pubescent plants	0	20	19	9
Gray-pubescent plants	5	4	2	0

Another cross was made between a self-colored brown with tawny pubescence and a Mandarin. In the F_2 generation tawny and gray-pubescent plants occurred in a 3:1 ratio. Plants with mottled and self-brown seed also occurred in a 3:1 ratio, making a 9:3:3:1 ratio when all characters were considered. All the gray-pubescent plants again showed the light-brown pigmentation, and tawny pubescence was associated not only with a darker brown pigment but also with a greater degree of mottling. The results obtained were as follows:

	Slight mottling	Medium mottling	Self-brown
awny-pubescent plants	5	35	10
ray-pubescent plants	9	1	6

It is not known whether the pigment in Mandarin seed coats is different from that of tawny-pubescent varieties, but abnormal Mandarin plants have been referred to which have developed a pigment nearly as dark as any brown variety. It seems entirely possible therefore that the difference in color may be due to the fact that the pigment is more concentrated in the seed coats of the tawny-pubescent plants. For this reason it seems logical that the factor *T*, for tawny pubescence (*13*), affects not only the shade of color but also the quantity of pigment formed.

Attention has already been called to the fact that Mandarin is more sensitive to certain types of environment than are some other varieties; so it can not be assumed that results similar to those just described would always be obtained. In some instances Mandarin has produced just as much mottling as Ito San or any other tawny-pubescent variety.

CROSS-FERTILIZATION

Piper and Morse (*14*) have pointed out that mottling may result from hybridization. All the crosses made by the writer between mottled and self-colored varieties confirm this statement. Self-colored black or self-colored brown seeds invariably have been recessive to yellow or green. A single pair of factors has been involved in all cases, but the dominance has been noticeably incomplete.

Figure 4, D, shows the typical coloration in the seed coats of an F_1 plant from a black × Mandarin cross. The black pigment was dominant to the brown carried by the Mandarin, but the seeds borne by the F_1 plant were generally badly mottled with black. This mottling was much more extensive than the brown ordinarily produced by Mandarins.

In an F_2 population from a cross such as the one just described, plants were produced with mottled or self-colored seed coats and the pigment was either black or brown, depending on the presence of R_1, the factor for anthocyanin pigment. Among the mottled seeds such striking variations in the amount of mottling have been observed that they can hardly be accounted for by environmental conditions.

Further study is necessary to explain these extreme variations in F_2 populations. It is possible that modifying factors may affect the expression of the single inhibiting factor. But as already mentioned, a heterozygous plant of the type resulting from a yellow × black cross is usually more mottled than the yellow parental variety. This complicates the situation in an F_2 population because it is possible that the plants with very badly mottled seed may simply be heterozygous for a single factor.

Extent of Natural Hybridization

In examining plants for mottling, the writer has occasionally chanced on a natural hybrid. During the last three years six plants have been found that might be classed as such, but in the course of

these observations over 20,000 plants have been examined; so the actual percentage of natural hybridization, although it occurs, is surely very low.

Woodworth (*17*), in a specially planned experiment, found that cross-pollination between two soy-bean varieties growing close together was approximately 0.16 per cent, but a somewhat higher percentage has been reported by Piper and Morse (*14*). The writer has not carried on a test comparable to Woodworth's, but from 15 plants with green cotyledons growing 3 feet from a variety with yellow cotyledons 5,500 seeds were obtained, 6 of which had yellow cotyledons, showing that they were the result of hybridization. This means that 0.11 per cent of the ovules had been cross-fertilized by pollen-carrying factors for yellow cotyledons; multiplying this figure by four to allow for the other possible combinations which would not be observed, gives 0.44 per cent.

The writer has not observed that honeybees or wild bees visit soy-bean flowers with any great frequency. Moreover, practically all of the flowers are fertilized before opening. This being the case, thrips are the most likely agency for the small amount of crossing that has been observed. Several species of thrips have been seen in soy-bean flowers and sometimes they are very numerous at flowering time. Frequently they have been seen making their way through the corollas of flowers before the pollen is ripe.

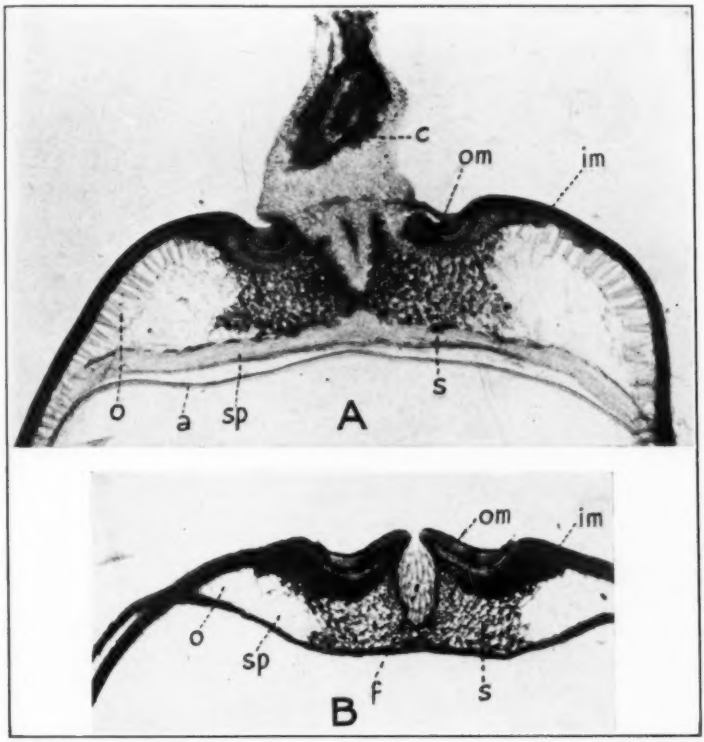
DISCUSSION

From the data presented in this paper, as well as from the results obtained by Hollowell [10] in Iowa, it is apparent that mottling is in some cases due to environmental factors. As a rule plants grown on a rich soil and spaced 3 feet apart produce badly mottled seed. Certain physiological abnormalities, however, have been responsible for the most extreme development of pigment.

Large seeds are produced on rich soil when plants are spaced 3 feet apart, but many cases have been cited where mottling has been associated with very small seed. The plants grown on a low-nitrogen nutrient solution furnish a striking example of this coincidence. Therefore, the accumulation of a large amount of synthetic material by the plant can not be the sole cause of mottling. On sandy soil thrifty plants have been grown, and large seeds entirely free from mottling have been produced.

It seems possible that the maturation of the seed may be a very important factor in the production of mottling. As long as the seed is growing a natural place is provided for the accumulation of synthetic material; but anything that causes premature maturation of the embryo, as when certain abnormal physiological conditions prevail, may bring about an accumulation of food material in other parts of the plant. Since mature cotyledons could not make use of these food materials, there would be an accumulation somewhere if the plant persisted in the process of photosynthesis. An accumulation in the seed coat under these conditions would explain how mottling may be brought about, and the development of pigment due to this cause corresponds very closely with the development of pigment by decortication, because in each case there is an accumulation of synthetic material in the tissue where the pigment is formed.

[10] HOLLOWELL, E. A. FACTORS INFLUENCING THE MOTTLING OF THE SOY-BEAN SEED COAT. 1924. [Unpublished master's thesis. Copy on file library, Iowa State College, Ames.]



Cross sections of hilum of soy beans, showing how the pigment is distributed in the seed coat. There was no staining, so all coloration is due to the natural pigments.

A, cross section of hilum from seed produced by hybridization with the Soysota variety.

B, cross section of hilum from the Wisconsin Black variety. This is the normal type of hilum for most varieties and it is dominant over the above type in inheritance: im, inner Malpighian cells; om, outer Malpighian cells; o, osteosclerid cells; c, remains of funiculus; sp, spongy parenchyma; s, asteroid parenchyma; f, fibrovascular bundle of testa; a, aleurone layer.

The study of mottling in relation to the position on the plant is particularly illuminating because it indicates that pigment formation may be controlled by a very delicate balance in nutritional conditions. The fact that seeds in the same pod always tend to be alike in spite of a wide variation within the plant emphasizes the importance of this very delicate balance.

The part played by restriction factors is not entirely clear, although observations indicate the presence of an enzyme or some active principle which inhibits the development of pigment. If such an enzyme is constantly at work preventing the formation of mottling pigments, any addition of sugars, the raw material from which these pigments are probably formed, would give this restriction mechanism an extra task. Considering the problem in this light, the whole question of mottling may well be reversed. Instead of inquiring about the factors responsible for mottling, why not ask, What are the inhibiting elements that prevent pigment formation?

Some strains of soy beans consistently produce more mottled seed than others, but the relative difference between two varieties may not always be the same. It has been found that a particular environment may affect some varieties differently. The Mandarin variety is not adapted to poor soils, especially heavy clays. Since the whole growth of the plant is more markedly affected by this kind of environment than are some other varieties, it is not surprising that mottling should also be affected to a greater extent. Such has actually been found to be the case. In 1924 the Mandarin plants grown on heavy clay soil were much more free from mottling than Manchus and Ito Sans grown on the same soil, a difference not so noticeable on richer soil.

In behavior, mottling is very much like the production of anthocyanin pigments in many other plants. Numerous factors may be involved, but the most important factor in the development of anthocyanin pigments is always heredity. Hereditary factors may entirely control the formation of pigment if the plant succeeds in growing at all. In other cases hereditary factors simply make it possible for the pigment to develop; and still other instances are known where no pigment ever develops, corresponding to albinism in animals. Borrowing the term "albinism" with this shade of meaning, we might say that there are no soy beans with "albino" seed coats as far as is known. All varieties apparently have the power to develop a certain amount of mottling under the proper conditions.

Where heredity alone is the controlling factor, other factors conducive to pigment formation, although still active, are obscured. Black and brown varieties of soy beans have such hereditary factors because they breed true and produce uniform seed regardless of environmental conditions.

When certain environmental factors are necessary in addition to heredity, pigment formation is seen to be extremely complicated. Pigmentation may be brought about or it may not, a particular combination of factors other than heredity being necessary. Various environmental factors are important, but we find that some are more important than others. Emerson's (*1*) sun-red colors in maize are primarily dependent on light. Although these pigments will not form without sunlight, other environmental factors are also important.

Of the different factors that influence mottling in soy beans it is difficult at present to designate any one as the most important. A rich soil and a large supply of synthetic material for each seed is important, yet very small seeds may be badly mottled. On certain types of soil mottling has been entirely determined by the distance of spacing, but the rich soil in 1925 caused mottling even on closely spaced plants. Since the mottling pigments are formed toward the period of maturity an additional factor comes in. If maturation is brought about in a certain way the seed may be nonmottled, but if the plant continues to live after the seeds are mature the seed coats may be entirely black or brown.

SUMMARY

All varieties of the soy bean (*Soja max*) with yellow or green seed coats included in this study proved to be subject to mottling. A study of hereditary and environmental factors showed that both were important. Certain selections consistently produced less mottling than others, but badly mottled seed has been produced from selections where mottling was least expected by supplying favorable environmental conditions for the formation of pigment in the seed coat.

The black and brown pigments responsible for mottling were found to be glucosides, and an explanation of their production by means of an accumulation of sugars has been considered. The most striking evidence for this theory was obtained from the observation that mottling was greatly increased by reviving the growth of the plant after the seeds were practically mature.

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PIGEON PEA ANTHRACNOSE [1]

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INTRODUCTION

The pigeon pea, or gandul (*Cajan cajan* (L.) Millsp.) (*Cajanus indicus* Spreng.), is an important food crop in Porto Rico. The pods are picked before they mature, and the fresh, green seeds have a high nutritive value and may be found in the local markets almost throughout the year. The leaves and pods are eagerly eaten by all classes of livestock, and the crop is highly recommended as forage for cattle.

During the past three years pigeon peas at the station have been considerably damaged by a spotting of the pods, accompanied by molding and decay of the seeds. In general appearance the disease is strongly suggestive of bean anthracnose, and the term "anthracnose" was applied to it even before the causal organism was determined. Examination of the available literature failed to reveal any reference to a pod disease of pigeon peas, and the serious damage wrought by it seemed to warrant investigation to determine the cause.

The disease is rather generally distributed over the island, especially in the western section. Infected pods were received also from Fajardo, on the eastern coast, where the disease is said to have caused considerable loss.

SYMPTOMS OF THE DISEASE

Anthracnose of pigeon pea may be recognized by the appearance of spots or blotches on the leaflets, pods, and seeds. On mature leaflets the spots are dark brown, definite, somewhat angular, and usually delimited by veinlets. The spots are most conspicuous on the superior surface of the leaflet. With age the spots become reddish brown and often slightly zonate, sometimes coalescing and covering a large portion of the leaf surface. Single spots range in diameter from 2 to 7 millimeters. Infections occurring during the rainy season may cause spotting on any part of the leaf blade, whereas infections occurring during dry weather usually affect the midvein and extend into the leaf tissues on both sides. The spots are very similar to those caused by *Vellosiella cajani* (Henn.) Rangel (*Cercospora cajani* Henn.), and a microscopic examination is often necessary to distinguish between them.

Infection of very young leaflets usually results in a blackening and shriveling of the veins, resembling lesions on *Phaseolus vulgaris* infected with *Colletotrichum lindemuthianum* (Sacc. and Magnus) B. and C. The leaves fall prematurely.

Circular to irregularly oval spots are formed on the pods. At first the spots are almost black, but later the center turns a dark brown and becomes surrounded by a narrow very dark purplish border.

[1] Received for publication Dec. 11, 1926; issued May, 1927.

(Fig. 1.) When moisture is abundant the centers become salmon colored and mealy in appearance, owing to the formation of masses of conidia. The pod shows no cankering or pitting such as occurs with infection of garden beans by *Colletotrichum lindemuthianum*, probably because of the tough, chartaceous character of the pigeon pea. Infection of very young pods results in shriveling, twisting, and deformation, and in most cases the pods die and drop.

Injury of the seed varies often in the same pod from a shriveling caused by pod infection in the region of the placenta to complete decay resulting from direct invasion by the fungus. In the latter case white tufts or a covering of white cottony mycelium usually may be seen. (Fig. 2.)

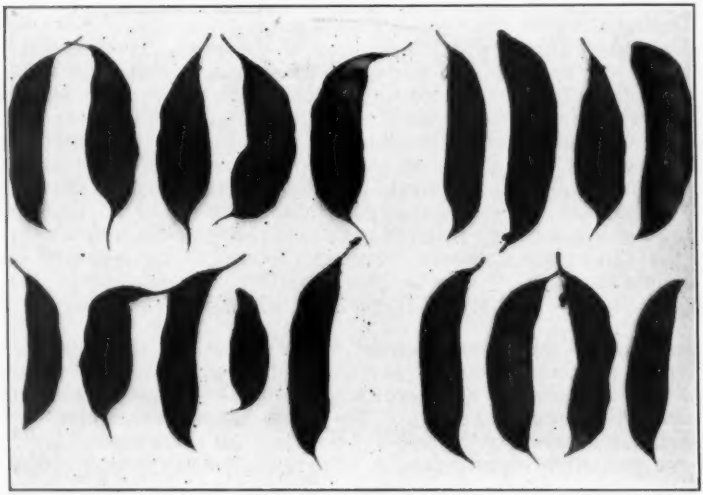

FIG. 1.—Left, pigeon-pea pods infected by anthracnose due to *Colletotrichum cajani*. The spotting and distortion of the pods is the most conspicuous symptom of the disease. Right, healthy pigeon pea pods

LOSSES CAUSED BY THE DISEASE

Damage attributable to anthracnose varies with the purpose for which the crop is to be harvested. When the pods are picked in the green stage the loss is much smaller than when they are allowed to ripen on the plant. Early in the dry season of January, 1925, 629 green pods were picked at random from plants in the station grounds. Of these 544, or 86.5 per cent, were found to be infected with anthracnose. The pods were mostly at the green-pea stage, at which time they are edible. The peas were shelled and graded as marketable or unmarketable; that is, discolored, moldy, decayed, or aborted. Table 1 shows the number of marketable seeds in diseased and healthy pods.

The 544 diseased pods yielded 1,342 marketable seeds; whereas, from the same number of healthy pods a yield of 2,118 seeds would be expected. The loss attributable to the fungus is 776 seeds, or 36.6 per cent.

In addition to the above easily calculated loss, there is the loss of young infected pods, which fall soon after the blossom drops. No exact data have been obtained on this kind of loss, but it is certainly considerable during the rainy season.

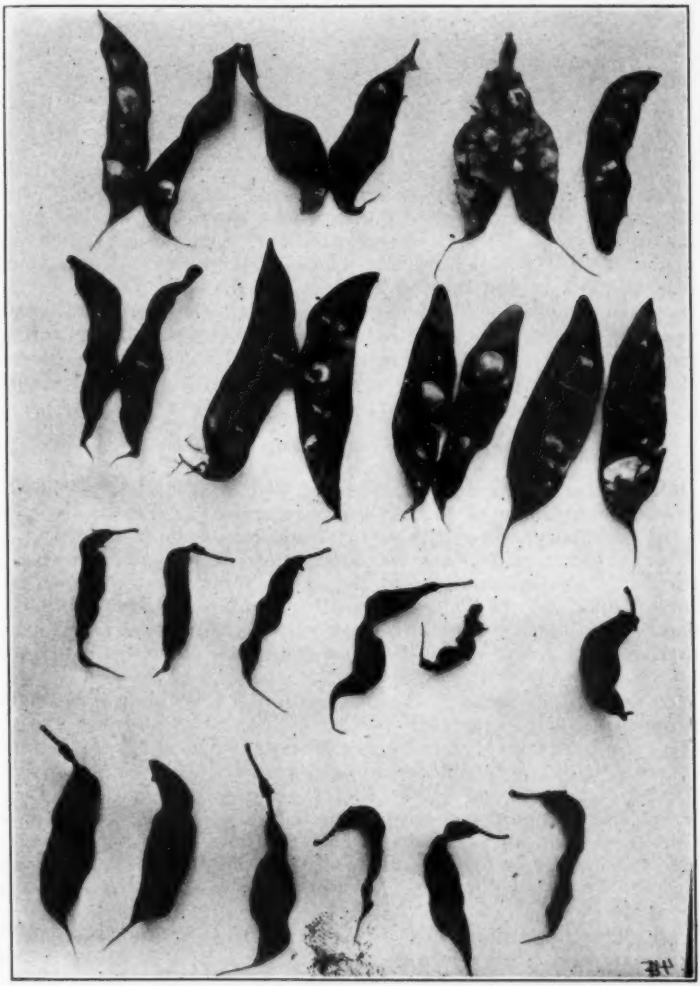

FIG. 2.—Above, split pigeon-pea pods bearing anthracnose spots. Note the white cottony mycelium. Some of the seed infection is confined to one or two dissepiments. Below, pods shriveled, distorted, and dead; the result of early infection by the anthracnose fungus

Probably the most important of the factors that determine the quantity of infection is rainfall. Pods which have formed during the rainy season show a much higher percentage of infection than those produced under dry conditions. Plants in sheltered situations produce a larger percentage of infected pods than plants to which the sunlight and breezes have free access.

TABLE 1.—*Marketable seeds in healthy and diseased pigeon-pea pods*

Marketable seeds per pod	Diseased pods in each class		Healthy pods in each class	
	Number	*Per cent*	*Number*	*Per cent*
0	66	12.1	0	0
1	84	15.4	0	0
2	101	18.5	0	0
3	144	26.5	21	24.7
4	121	22.3	52	61.2
5	28	5.2	12	14.1
Total	544	100.0	85	100.0

Although the pods may become infected during any green stage, the resulting damage is greatly affected by the age of the pod at the time of infection. Early infections before the pod is 1 inch long usually cause the death of the whole pod. Later infections may cause the loss of the seed in the infected dissepiments only. The dissepiments of the pod are closed by the constrictions, and usually the fungus invades the seed in dissepiments on which acervuli are borne, but does not pass the constrictions. Late infections produce dotlike acervuli which do not enlarge much, and usually the fungus fails to penetrate to the interior.

THE ORGANISM

Microscopic examination of diseased pods and leaflets reveals the presence of a fungus of the anthracnose group. Acervuli are borne on the pod spots and on the superior surface of the leaf spots.

The conidia from pod acervuli are cylindrical, broadly elliptical to irregular in shape, usually rounded at the ends, hyaline when very young, but later becoming densely granular, or few—guttulate, straight, or slightly curved. They are continuous or very rarely 1-septate, and are 12 to 17×3.5 to 7.2 microns, with an average of 13.6×5.6 microns. (Fig. 3, B.)

The conidia are borne in abundance on pod acervuli and in smaller numbers on the leaf acervuli.

The conidiophores are simple, hyaline, cylindrical or slightly swollen, usually septate near the base, rounded at the apex, and 15 to 25×3 to 5.5 microns. (Fig. 3, A.)

Setae are numerous on old pod spots, but occur only occasionally on the leaves. They are fasciculate, dark brown, cylindrical, usually somewhat curved or bent, rounded at the apex, 1 to 5 septate, and 70 to 120×3.3 to 4.2 microns, with an average of 100×3.5 microns. (Fig. 3, D.)

The above description of the fungus places it in the genus Colletotrichum. Two references to the occurrence of this genus on the pigeon pea have been found. Dash (*4, p. 38*) stated,[2] "A species of Colletotrichum was found on pigeon pea at Codrington [Barbados] which seemed to cause death to the branches attacked." Rangel (*6, p. 154–155*) in Brazil described and figured a Colletotrichum on leaf spots of the pigeon pea. His description follows:

[2] Reference is made by number (italic) to "Literature cited," p. 596.

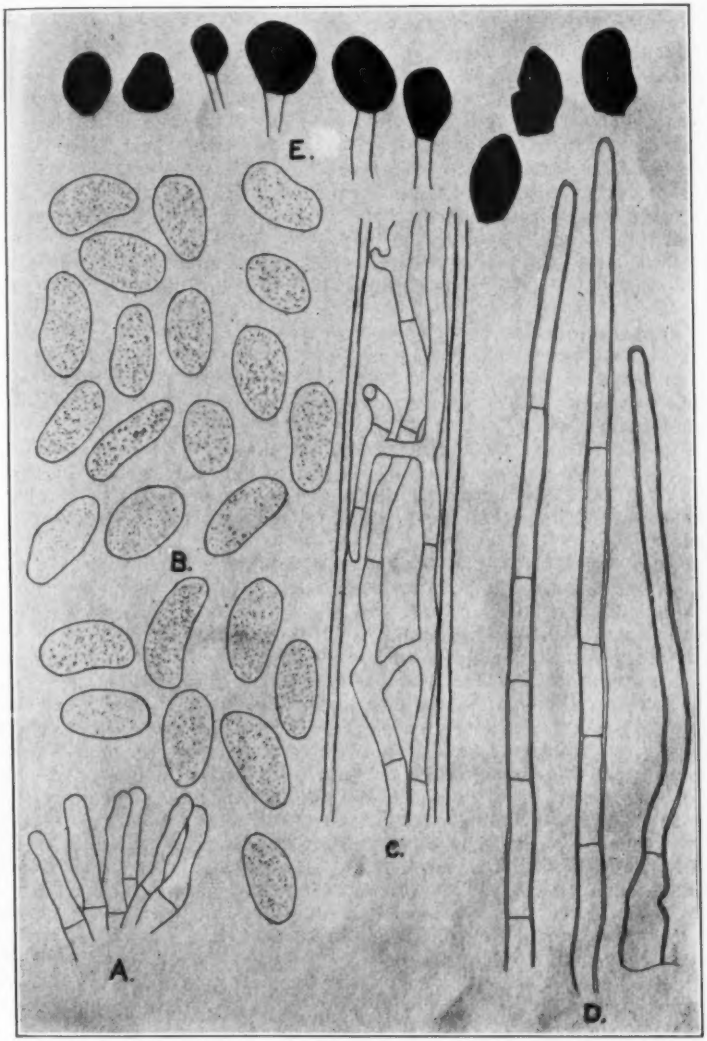

FIG. 3.—*Colletotrichum cajani*: A, conidiophores; B, conidia; C, section of a hair from an infected pigeon-pea pod, showing invasion by the mycelium of the fungus; D, setae from a pod acervulus; and E, appressoria from 2-months-old potato-dextrose agar culture. × 1175

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Colletotrichum cajani, Rangel (n. sp.).

Maculis majusculis, apicalibus, obscure ferrugineis, indeterminatis vel atro-brunneo marginatis; acervulis plerumque epiphyllis, minutis, olivaceis; conidiis cylindraceis, oblongis vel lato ellipsoideis, continuis, granulosis, hyalinis, 10—15=4—5μ setulis rectis vel curvulineis, septatis, brunneolis, 45—60=4—5μ. In foliis vivis *Cajani indici*. Niteroy. Brasiliae. (Exs. 723. Maio. 1913).

The fungus here described does not differ essentially from that under discussion. As to the differences in the size of conidia and setae, it should be remembered that Rangel's observations were made on material from leaf spots, whereas the writer used diseased pods on which the conidia and setae developed more profusely. The fungus under discussion is therefore referred to *Colletotrichum cajani* Rangel.

In culture the fungus grows rapidly on a variety of nutrient media. On potato-agar plates the growth is closely appressed, thin, white, spreading, regular, radiate and sliming from below. At 25° C. the mycelia after 72 hours average 22 millimeters in diameter, and after 120 hours, 37 millimeters. No spores were produced during one week.

On potato-dextrose agar plates the growth has the same characters as on potato agar except for a slight increase in density and in rapidity of growth. After 72 hours the mycelia average 25 millimeters in diameter, and after 120 hours, 46 millimeters. After one week no conidia were borne.

On oatmeal-agar plates the growth was intermediate in density and rapidity between that occurring on potato agar and potato-dextrose agar. After 72 hours the mycelia average 23 millimeters in diameter and after 120 hours, 43 millimeters. No conidia were borne in one week.

On steamed pigeon-pea pods a luxuriant growth of white cottony mycelium is made. Conidia are abundant after a week's growth. The conidia are very similar in size and shape to those borne on pods as the result of natural infection.

In 2-months-old cultures on potato-dextrose agar and pigeon-pea pods, appressoria, as described and figured by Edgerton (*5*) are abundant. They are dark brown in color and are formed by the swelling and thickening of the wall of an apical hyphal cell. They usually occur singly, but occasionally two to five apical cells become swollen, producing the appearance of a chain of chlamydospores. The appressoria are oval to irregular in shape and vary in size, ranging from 6.4 to 12 × 4.8 to 6.4 microns.

Edgerton (*5*) found appressoria occurring generally in the anthracnose fungi following the germination of conidia in a medium lacking nutrient material. In the writer's potato-dextrose agar cultures conidia were never numerous, yet appressoria appeared in large numbers, and observations indicate that at least a large majority were formed directly from hyphal cells. The resulting thick-walled cells or resting bodies seem in every respect to be analogous to chlamydospores.

INOCULATIONS

The fungus was grown on steamed pigeon-pea pods for two weeks, with profuse conidia production. A water suspension of conidia and mycelium was atomized on 107 pigeon-pea seedlings about 8 inches high. The seedlings were then watered daily by sprinkling and kept shaded for 48 hours. The earliest symptoms of infection appeared about five days later, when the veins in some of

the youngest leaves darkened. After 12 days infection was well advanced. Apparently the leaflets emerging from the bud during inoculation were most easily infected. Of 107 inoculated seedlings, 102 showed unmistakable symptoms of infection. In every case infection appeared on the veins of the leaflets, resulting in death in severely affected specimens. Deformed leaflets were borne by other seedlings as a result of the continued growth of the healthy portions and the arrested growth of infected veins. Infection of the older leaflets was not common. When infection did occur, the blades were spotted. The older leaflets showed no vein infection. Elongated narrow stem lesions were present on 54 seedlings. The lesions were as yet hardly more than a darkening of the epidermal cells. The woody character of the pigeon-pea stem prohibits the formation of cankers such as occur on anthracnose-infected garden beans. Petiole infections occured on 25 seedlings and usually caused the affected leaflets to yellow and fall.

The fungus was reisolated from vein, stem, and petiole lesions and was found to be identical morphologically and in cultures with the inoculating organism.

Thirty-eight seedlings were used as controls. These received the same treatment as was given the inoculated plants except that sterile water was substituted for the inoculating fungous suspension. Thirty-four seedlings remained healthy, two were doubtful, and two became slightly infected.

Garden-bean seedlings were inoculated like the pigeon-pea seedlings, the pigeon-pea seedlings being used for parallel inoculations as a check on the virulence of the inoculating cultures. The varieties of *Phaseolus vulgaris* used included Giant Stringless Green Pod, Valentine Pencil-Pod Black Wax, and Refugee (all bush beans). These varieties were selected because of their susceptibility to *Colletotrichum lindemuthianum*. Barrus (*1*) showed them to be susceptible to the alpha and beta strains, and Burkholder (*3*) demonstrated their susceptibility to the gamma strain.

No infection was obtained on the varieties named, although the parallel inoculations on pigeon peas produced abundant leaflet-vein infection.

The infection of varieties of *Phaseolus vulgaris* with a Colletotrichum from *Cajan cajan* could hardly be expected in view of the results obtained by Barrus (*2*). Summarizing the results of extensive inoculation experiments, he states: "There are evidently but few species outside the genus Phaseolus which are susceptible in any degree to anthracnose [*C. lindemuthianum*], and no plants except varieties of *Phaseolus vulgaris* are susceptible to such an extent that the disease becomes epiphytotic in regions where such plants are extensively grown."

SUMMARY

The pigeon pea (*Cajan cajan*) in Porto Rico is attacked by anthracnose, resulting in spotting of the pods and leaves and destruction of the seeds.

On young leaves infection occurs mostly on the veins and causes them to blacken and shrivel; on young pods infection causes distortion, abortion, and death.

The losses sustained are due to the destruction of young pods and the decay or discoloration of one to all the seeds in infected pods.

Infection is most serious during periods of heavy rainfall.

The causal organism is referred to *Colletotrichum cajani* Rangel, which is now first recorded as the cause of a pod and seed disease.

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